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# A METHOD OF SEPARATING THE ANTERIOR PITUITARY-LIKE HORMONE FROM THE URINE OF PREGNANT WOMEN

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It has been demonstrated that the anterior pituitary hormone plays an important part in the estrous cycle of animals. By analogy and certain clinical observations, a similar situation probably exists in the menstrual cycle of the human. Since the anterior pituitary hormone or some substance with similar action occurs in the urine of pregnant women, it was considered that a similar substance might exist in the urine of the normal menstruating woman, probably at the time of ovulation. If this could be proved, it would indicate that the anterior pituitary hormone does play a part in the menstrual cycle.

The accepted Friedman modification of the Zondek-Aschheim test is not sensitive enough to demonstrate the presence of the hormone in the urine during the menstrual cycle. The problem resolves itself into developing a method of concentrating the hormone from large volumes of urine in sufficient amounts to give a positive ovulation test. In the procedures incident to the development of the method, new properties of the hormone were noted, and a method for preparing potent extracts was evolved. It is this method applied to the urine of pregnancy and these properties which we wish to report at this time.

Because of the extreme lability and solubility of the hormone, its separation from the undesirable constituents in urine has been difficult. Earlier workers in the field have prepared extracts from the anterior pituitary gland, placenta, and urine, all of which have similar physiological properties. Zondek and Aschheim (1, 2) have prepared the hormone from urine of pregnancy by

acidifying the urine with acetic acid, filtering, and concentrating the filtrate to a small volume at 40°. To such concentrates 95 per cent alcohol was added and the precipitate formed dissolved in water for subsequent use. Biedl (3), Evans and Simpson (4), Reiss and Haurowitz (5), and Wallen-Lawrence and van Dyke (6) have all prepared extracts based on the Zondek-Aschheim method. Reiss and Haurowitz (5) and Fischer and Ertel (7) prepared the hormone by means of adsorption on kaolin and elution with dilute ammonium hydroxide. Recently Katzman and Doisy (8) have advocated the adsorption and elution method. In one of their methods the hormone is adsorbed on charcoal or norit, eluted with 90 per cent phenol, and the hormone separated from the phenol by solution of the latter in alcohol. In the other preparation the hormone is adsorbed on benzoic acid, and the latter freed from the hormone by solution in alcohol or acetone.

Other methods of preparation have been advocated based on the precipitation of the hormone by various substances such as uranyl acetate (Reiss and Haurowitz (5)) and an elaborate method by Weisner and Marshall (9) in which the urinary proteins are thrown down with sulfosalicylic acid, the filtrate concentrated, phosphates and sulfates removed with barium hydroxide, and the resulting solution treated with alcohol to precipitate the hormone. Another method by the same authors is based on the precipitation of the hormone with phosphotungstic acid, and the extraction of the hormone with dilute ammonium hydroxide and final precipitation with alcohol. Since we are particularly interested in a method by which we may estimate quantitatively the amount of hormone present in the original sample of urine, none of these latter procedures was entirely satisfactory.

*Experimental Animals and Material Used*—In all the following experiments the virgin female rabbit weighing 5 to 6 pounds was used as the test animal. The various extracts were injected intravenously at 4 to 5 p.m. and the following morning, 18 to 20 hours after injection, a laparotomy was performed under intraperitoneal amytal anesthesia. A positive ovulation test, according to the Friedman (10) modification of the Zondek-Aschheim test, was taken to indicate the presence of anterior pituitary-like hormone. The animals were housed and cared for under stand-

ard conditions according to the method noted by Wilson and Corner (11). Only healthy animals were used in the final analysis of a preparation, since it has been noted that marked infection, disease, or debility nullifies a positive result when the minimum amount of a standardized preparation is used. Such animals often require 2 to 3 times the minimum dose to effect ovulation. Older and less healthy animals were used only to test new extracts for toxicity or fatality.

The experimental work which follows was done with standardized urine of pregnant women, which consisted of composite urine as obtained in our Outpatient Department, and was injected intravenously into the experimental animal in graded doses until the smallest amount necessary to cause ovulation in at least six rabbits was determined. This amount of urine was called 1 rabbit unit, and such a batch of urine designated as standardized urine of pregnancy. The concentration of the anterior pituitary-like hormone varied in the several batches of urine of pregnancy, and as a result the number of rabbit units per cc. varied so that the rabbit unit and not cc. formed the basis for comparison.

#### *Methods for Separation and Concentration of Hormone*

*Method 1*—Several methods were tried for isolating the hormone in concentrated form. Since the hormone is reported heat-labile at 60°, concentration at reduced temperature and pressure to a small volume should theoretically give a preparation suitable for our purpose. In order to investigate this, several batches of 500 cc. each of standardized urine of pregnancy were concentrated on a water bath, at not above 35° and 18 to 25 mm. of mercury, to one-tenth or less their original volume, the process requiring from 2 to 6 hours. To aid in the concentration by lowering the surface tension, standardized urine in some instances was mixed with enough 95 per cent alcohol to bring the concentration to about 20 per cent. Concentrates so obtained were often very toxic or fatal to the experimental animal. Four concentrates which were non-toxic were found to have lost roughly about 60 to 75 per cent of the hormone on the basis of the original standardized urine. Obviously such concentrates could not be used for experimental study. Precipitation of such concentrates according to the method of Zondek and Aschheim (1, 2) and



Evans and Simpson (4) gave preparations which, for the most part, were non-toxic, but occasionally toxic or fatal preparations were obtained. By so precipitating the concentrates with alcohol or acetone, more of the hormone was destroyed, the amount of which was not definitely determined. The toxic preparations were washed or refluxed with ethyl ether, but this apparently did not lessen the toxicity to any marked degree. The toxic substances in such preparations are not precipitated by ammonium sulfate, but if this further step is added to the purification of the hormone, more of the hormone is destroyed or lost.

*Method 2*—Since in our intended studies only daily samples of urine were to be used, precipitation of the hormone by the addition of 5 volumes of alcohol, while cumbersome, could be used. Using total daily outputs of urine of pregnancy and urine from non-pregnant women and precipitating with alcohol gave precipitates which, when dissolved in small amounts of water, were often toxic or fatal to the experimental animal. Such a procedure with four different batches of standardized urine of pregnancy destroyed from 40 to 75 per cent of the hormone in those extracts which were non-toxic. Reprecipitation by dissolving the precipitate in water and adding alcohol or acetone does not entirely remove the toxicity, and such a step destroys more of the hormone, the exact amount of which has not been determined. As the hormone is not precipitated by 2 volumes of 95 per cent alcohol, and since 2 volumes of alcohol added to urine precipitates considerable undesirable material, urines were so treated and filtered, and to the filtrate 3 to 4 more volumes of 95 per cent alcohol were added. This procedure did not remove any of the toxic material.

*Method 3*—Saturating standardized urine with ammonium sulfate gave a flocculent precipitate within an hour. This was filtered off, dissolved in water, and the resulting solution injected, no further purification, according to the method of Dickens (12), being used. 60 to 70 per cent of the hormone was lost or destroyed by this procedure. None of the preparations so prepared was toxic to the experimental animal.

*Method 4*—Reiss and Haurowitz (5), Fischer and Ertel (7), and recently Katzman and Doisy (8) have noted that the hormone can be adsorbed on such materials as kaolin, charcoal, or norit. With standardized urine of pregnancy and charcoal or kaolin,

attempts were made to determine whether the hormone could be quantitatively adsorbed and extracted, and whether toxic materials were contained in the resulting product. To known volumes of standardized urine 10 per cent, by weight, of powdered willow charcoal or kaolin was added, and at definite intervals varying from 1 to 16 hours the charcoal was separated from the urine by centrifugation. The supernatant urine, supposedly free from the hormone, was injected intravenously in 5 to 10 cc. quantities. After 2 hours adsorption this urine was positive in two cases; after 4 hours adsorption, positive in four cases; but after 16 hours adsorption, negative in four cases. 4 hours time proved insufficient for complete adsorption, but after 16 hours in the refrigerator complete adsorption was obtained, no injections being made between 4 and 16 hours. Several batches of standardized urine of pregnancy, 0.75 cc. of which was necessary to cause ovulation, were negative in 10 cc. quantities after treatment with charcoal overnight in the refrigerator. As will be demonstrated below, the hormone can be extracted with 0.1 to 0.25 N NaOH and the resulting solution contains roughly 50 per cent of the original hormone, is non-toxic to rabbits, and concentrated 5 to 10 times.

In the above methods, it has been shown that: (1) Heat concentration causes a loss of or destroys 70 per cent of the hormone, and the product is toxic in most cases. (2) Alcohol precipitation causes a loss of or destroys 40 to 75 per cent, and the product is toxic in most cases. (3) Ammonium sulfate concentration causes a loss of or destroys 60 to 70 per cent, and the concentrate so prepared was non-toxic. (4) Adsorption and extraction yield 50 per cent of the hormone, and the product is non-toxic. Thus it was concluded that the adsorption and extraction method was the most suitable, and further studies were made to improve the technique of this method.

*Studies on Adsorption Method*—Various quantities of kaolin or charcoal from 0.5 to 10 per cent were added to equal volumes of standardized urine of pregnancy, and after standing in the refrigerator overnight with occasional shaking, the charcoal was separated by centrifugation and the supernatant urine injected in 10 cc. amounts. It was found that 1.5 to 2.5 per cent would adsorb all the hormone. In all our work we used 2 to 2.5 per cent charcoal.

Since adsorption depends upon salt concentration, surface tension, and hydrogen ion concentration, by altering any one of these the hormone should be freed. Dilute alcohol and acetone were tried without avail. Various salts, as monosodium phosphate, disodium phosphate, sodium carbonate, sodium bicarbonate, and sodium chloride, gave negative results. Attention was then turned to hydrogen ion concentration. A series of buffered solutions varying from pH 1 to pH 13.2 was prepared. The standard sodium acid phosphate-sodium hydroxide, acetic acid-sodium acetate, and glycoll-sodium hydroxide mixtures were used. Samples of charcoal adsorbing the hormone were treated with these buffered solutions, and all gave negative reactions except pH 13.2 which is 0.1 N sodium hydroxide. Ten samples of charcoal adsorbing the hormone from various batches of standardized urine, were extracted with 0.1 N NaOH. All gave positive reactions. Normalities of sodium hydroxide from 0.1 to 0.0125 N were tried, and it was found that the hormone could not be extracted below a normality of 0.025 N. By using equivalent amounts of the same charcoal adsorbing the hormone and equal quantities of the various normalities of NaOH, negative reactions were obtained in two cases at 0.0125 N; positive reactions in two cases at 0.025 N; positive reactions in four cases at 0.05 N; and positive reactions in four cases at 0.1 N NaOH.

Apparently the hormone is stable in alkali and its extraction can be effected from kaolin or charcoal by sodium hydroxide. Since one gains the impression from some of the earlier literature that the hormone is unstable in alkalies, as noted by Bugbee *et al.* (13), and since we could effect extraction by sodium hydroxide, enough solid sodium hydroxide was added to the standardized urine of pregnancy to make it equivalent to 0.05 N sodium hydroxide. Quantities of this alkalinized urine equivalent to 1 rabbit unit of the original standardized urine were injected intravenously into the rabbit, and the findings indicate that the hormone is not destroyed after a period of 24 hours. Conversely, enough concentrated hydrochloric acid was added to a similar quantity of a standardized urine of pregnancy to make the urine equivalent to 0.1 N, and this injected intravenously shows that the hormone was in no way destroyed.

The method as now used consists of adding to urine of preg-

nancy 2.5 per cent, by weight, of powdered willow charcoal or kaolin after adjusting to hydrogen ion concentration of 4.2 with brom-phenol blue, allowing to stand in the refrigerator overnight with occasional shaking, centrifuging, washing the charcoal or kaolin with a small quantity of distilled water, and extracting the hormone by treating with 0.1 N sodium hydroxide in the refrigerator overnight. The charcoal is separated by centrifugation and the supernatant extract injected. Filtration through filter paper and asbestos is avoided, since it has been determined in a few instances that a part of the hormone is adsorbed. The yield of the hormone by this method is 50 per cent. Recent work with hydrogen ion concentration indicates that the yield of the hormone depends upon the pH at which it is adsorbed, extracted, and injected. Studies on this phase of the problem are in progress now.

*Properties of Adsorbed Extracted Hormone*—If to the alkaline extracted solution of the hormone or any of the neutralized solutions down to a hydrogen ion concentration of 1, 5 volumes of alcohol or acetone are added and the mixture allowed to remain in the refrigerator at 0° overnight, no precipitate is formed. If ammonium sulfate is added to the alkaline extracted solution of the hormone to the point of saturation, no precipitate is formed. The inference drawn is that the hormone free of urine is not precipitated by alcohol, acetone, or ammonium sulfate but in urine is carried down with other precipitable substances present, and that the hormone in the true sense of the word is not precipitated by any of these reagents. Wallen-Lawrence and van Dyke (6) noted this in their preparation. However, since in the purification of their product they used norit to remove the color of their preparation, no doubt most of the hormone was adsorbed.

By separating the hormone from the urine of pregnancy by adsorption on kaolin or charcoal and extracting with 0.1 N NaOH, the aim has been not only to prepare concentrated extracts but to recover the hormone quantitatively. Concentrated potent extracts have been made so that 1 cc. is equivalent to 5 to 8 rabbit units.

Both the biuret and Millon tests applied to these fairly concentrated solutions have been negative. Most authors have reported positive reactions, but Zondek and Aschheim (1, 2) have reported

negative reactions. Perhaps the method of preparation noted by other authors precipitates proteins along with the hormone or our preparations are not concentrated enough to give a positive reaction.

Various immiscible solvents, as butyl alcohol, caprylic alcohol, ethyl acetate, have been tried in an effort selectively to dissolve the hormone, but all attempts have been negative.

#### DISCUSSION

A systematic study of available methods has revealed that the hormone is extremely sensitive to even the most simple of procedures, and that it is easily completely or partially destroyed or rendered inert by such procedures as heating and precipitations by alcohol or ammonium sulfate. These procedures destroy 50 to 75 per cent of the hormone and the resulting product in most cases is toxic. Many authors have prepared very concentrated preparations, as noted by Katzman and Doisy, and of the various methods tried, that of adsorption and extraction seems most satisfactory. A more extensive survey of the effect of hydrogen ion concentration on the extent of adsorption and extraction is in progress.

In our studies we have noted that in fairly concentrated preparations the biuret and Millon tests are negative, a finding agreeing with that of Zondek and Aschheim but contrary to others. Our alkaline preparations have been repeatedly injected intravenously into the rabbit, and, except in a few instances, are extremely well tolerated. By the method of adsorption and extraction, foreign proteins are apparently not a contaminant, or the hormone, if it is protein in nature, is in too dilute a solution to give a positive reaction. Since the hormone is not precipitated from the alkaline extracted solutions or alkaline extracted solutions which have been neutralized to various hydrogen ion concentrations by alcohol, acetone, or ammonium sulfate, and since these reagents will precipitate proteins, the evidence would seem to indicate that the hormone is not protein in nature.

Since toxicity is always a factor to be avoided, we have noted that the toxic material cannot be removed by ether, that it is removed by  $(\text{NH}_4)_2\text{SO}_4$ , and that adsorption and extraction of

the hormone from toxic urine does not carry any of the toxic material to the final preparation.

The process of adsorption and extraction is dependent upon hydrogen ion concentration. Apparently adsorption is best effected at pH 4 and extraction at pH 12 to 13. Further work is being carried on along these lines. The hormone was found to be soluble in 0.1 N NaOH and 0.1 N HCl, and to be unaffected by these solvents over a period of 24 hours.

#### SUMMARY

1. A method for the preparation of the anterior pituitary-like hormone from urine of pregnancy, based on adsorption and extraction, is described. The process is apparently dependent on hydrogen ion concentration. The adsorbent removes the anterior pituitary-like hormone quantitatively.

2. Such preparations give negative biuret and Millon tests.

3. The active material after adsorption and extraction from urine of pregnancy is not precipitated by alcohol, acetone, or ammonium sulfate. Use of these reagents destroys a greater part of the hormone.

4. The active material is extremely sensitive. Quantitative data as to its lability are noted.

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## THE CALCIUM AND POTASSIUM CONTENT OF DOG TISSUES AND THE INFLUENCE OF THYRO- PARATHYROIDECTOMY\*

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The present research was undertaken to study the calcium and potassium of tissues in a condition of parathyreopriva, one in which the calcium is markedly reduced, and in which opportunity is afforded for disturbance of the Ca:K ratio in a tetanic muscle.

MacCallum and Voegtlin (1) analyzed the brains of three dogs in parathyroid tetany, and found a reduction of calcium. Parhon, Dumitresco, and Nissipesco (2) analyzed the brains of parathyroidectomized dogs and cats, and found values very variable. Behrendt (3) states that the potassium of muscle of parathyroidectomized dogs is increased, and the calcium is constant. He gives no figures.

Forster (4), Voit (5), Katz (6), and Kapeller and Kutschera-Aichbergen (7) determined the calcium along with other mineral constituents in the tissues of man and normal domestic animals, and found wide variations in the calcium content. Their findings are in accord with the work of Rona and Heubner (8) on normal cats, and that of Barral and Barral (9) on rabbits.

The work of Katz (6) and Lederer and Stolte (10) on the potassium content of the tissues of normal animals shows the content of this element rather constant as compared with that of calcium.

### *Procedure*

After a control period of several days in the laboratory the thyroid glands of dogs were removed under light ether anesthesia.

\* The experimental data presented in this paper were taken from the thesis submitted by T. C. Jaleski to the Faculty of Yale University, School of Medicine, in candidacy for the Degree of Doctor of Medicine, 1931.

† Deceased.



Aberrant parathyroids were searched for and also removed. All animals were killed at the height of tetany by section of the femoral artery and tissue samples were taken immediately.

TABLE I  
*Calcium and Potassium Contents of Dry Tissues of Dog*

Dog No	Liver	Kidney	Heart	Lungs	Spleen	Brain	Muscle	Blood
Mg of Ca per 100 gm. of tissue of normal dogs								
I	14 25	27 11	19 38		23 51	32 57	15 15	8 09
VI	6 42	39 80	17 06	53 47	27 31	18 43	13 95	11 31
VII	4 13	36 32	15 80	49 47	24 36	8 02	10 50	11 12
Mg of Ca per 100 gm. of tissue of parathyroidectomized dogs*								
II	21 28	46 69	44 57	170 95	45 33	58 36	42 97	5 76
III	18 09	49 46	20 89	43 66	29 33	24 69	19 76	8 82
IV	16 39	31 83	16 49	34 78	19 26	15 67	11 14	6 20
V	5 73	34 89	12 88	234 80	22 86	58 44	16 74	6 13
VIII	14 33	41 38	10 70	33 01	19 71	170 60	11 66	6 95
X	6 38	32 25	12 33	41 63	22 75	33 48	14 02	6 35
Mg of K per 100 gm of tissue of normal dogs								
I	913 7	1198 5	1617 3		1951 0	1750 0	1113 6	17 10
VI	993 6	1350 4	1494 0	931 1	1621 1	1629 4	1684 4	19 50
VII	745 8	1333 8	1567 0	1081 9	1934 5	1735 5	1784 6	20 87
Mg of K per 100 gm of tissue of parathyroidectomized dogs*								
II	906 5	1092 5	1177 7	1758 1	1248 3	1281 2	1107 0	22 08
III	847 0	1114 0	2006 6	1393 2	1011 4	2138 3	1926 2	20 10
IV	792 7	815 9	1602 1	953 1	770 9	2018 1	1172 9	21 00
V	1152 7	1322 3	1554 3	788 3	1777 0	1771 5	1624 8	28 12
VIII	1024 1	1260 5	1616 5	1137 8	1630 5	1728 5	1695 7	26 33
X	949 4	1243 8	1452 5	1113 6	1451 2	1705 1	1804 0	24 18

\* Dogs killed in tetany.

*Method of Sampling*—All blood samples were taken from the jugular vein without the use of an anticoagulant. Tissues were prepared for analysis by charring in the presence of 4 N sulfuric acid on the electric hot-plate followed by complete ashing in the electric muffle. The ash obtained was extracted with dilute HCl

and made up to 25 cc. Aliquots were taken for the determinations which were made in duplicate.

For the determination of potassium in the blood the proteins from a 5 cc. blood sample were precipitated with 10 per cent trichloroacetic acid, and the filtrate was ashed and treated according to the method of Shohl and Bennett (11). The same method was used for tissues, 2 cc. aliquots being used.

For the determination of the blood calcium the Clark and Collip (12) modification of the Tisdall-Kramer (13) method was used. For tissues 20 cc. aliquots were evaporated to a small volume and treated according to the Tisdall-Kramer (13) method.

Water was determined by 4 day desiccation *in vacuo*.

#### DISCUSSION

The results, which are given in Table I, give no indication that there is any departure from normal calcium content in any of the tissues. The wide variations present could very well mask the slight changes in the tissue which would be necessary to account for the calcium disappearing from the serum, a matter of some 50 to 100 mg. depending on the size of the animal. The fact that even in the presence of large stores of calcium in the bone and tissues the serum calcium remains low, points to a disturbance of mobilization and absorption functions. Also a shift from the ionized to the un-ionized form or *vice versa* would certainly affect the amount of calcium found in the serum.

#### SUMMARY

1. Judging from the experimental investigation in dogs, there is no apparent change in the calcium and potassium contents of tissues after thyroparathyroidectomy.

2. The calcium content of the tissues of normal and parathyroidectomized animals is subject to wide variations.

3. In tetania parathyreopriva serum calcium is reduced and blood potassium is not.

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## OUABAIN

### II. THE DEGRADATION OF ISOOUABAIN

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(Received for publication, March 23, 1933)

In our previous communication<sup>1</sup> on ouabain, the first successful attempts to obtain a crystalline aglucone derivative of this glucoside after cleavage of the sugar were described. The aglucone itself has not been directly obtained from the glucoside because of the severe hydrolytic conditions necessary to cleave the relatively firm glucosidic union between the aglucone and the sugar, rhamnose. A remarkable cleavage was, however, produced, by a special procedure of acetolysis, of a derivative, dihydrodesoxyouabain heptacetate. Under these conditions the aglucone derivative displayed an unusual lability. Instead of an expected pentaacetate of dihydrodesoxyouabagenin, a triply unsaturated monoacetate,  $C_{24}H_{30}O_4$ , was obtained, which resulted from the simultaneous loss of 4 acetic acid molecules and 1 carbon atom as formaldehyde. After saponification, the corresponding unsaturated hydroxylactone,  $C_{22}H_{28}O_3$ , thus possessed 1 carbon atom less in its make-up than the skeleton of ouabagenin itself. This fact complicated future attempts to use this substance as a basis for its structural correlation with strophanthidin.

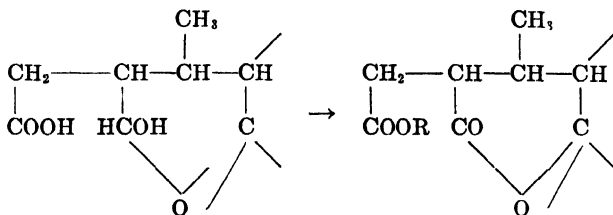
We have attempted, therefore, to make use of isouabain which we previously<sup>1</sup> obtained by the action of alkali on ouabain. But here again a similar phenomenon has been observed. Systematic attempts to hydrolyze isouabain, in order to obtain the isoaglucone, isouabagenin, proved futile. As in the case of ouabain itself, only unpromising resins were obtained. The preliminary acetylation of isouabain was then attempted at first with the zinc chloride-acetic anhydride method successfully used

<sup>1</sup> Jacobs, W. A., and Bigelow, N. M., *J. Biol. Chem.*, **96**, 647 (1932).

in the case of ouabain. However, only unworkable resins resulted. Acetic anhydride with sulfuric acid at room temperature was then tried. The reaction mixture yielded a substance which to our surprise proved to be rhamnose-free. A further study of the reaction showed the optimum condition for the formation of the substance to be at 70°.

The substance on investigation proved to be the *monoacetate of a trianhydrohydroxylactone*, with the formula  $C_{24}H_{28}O_5$ , and for saponification required 2 equivalents of alkali corresponding to a lactone group and an acetyl group. On reacidification relactonization gradually occurred with the formation of the *trianhydrohydroxylactone*,  $C_{22}H_{26}O_4$ . In acetic acid solution the monoacetate was found to absorb 3 mols of hydrogen, which showed therefore the presence of three double bonds. However, the substance proved unusually resistant to hydrogenation in neutral solutions such as in alcohol or ethyl acetate. Similarly, the hydroxylactone,  $C_{22}H_{26}O_4$ , failed to absorb hydrogen in ethyl acetate solution. Undoubtedly the positions occupied by the three double bonds are the cause of this resistance and recall our experience with trianhydrostrophanthidin.<sup>2</sup>

The hydroxylactone,  $C_{22}H_{26}O_4$ , on saponification gave an acid,  $C_{22}H_{28}O_5$ , which could not be crystallized. However, from this a crystalline *methyl ester*,  $C_{23}H_{30}O_5$ , was obtained. This ester on oxidation with chromic acid yielded a *trianhydroketolactone ester*,  $C_{23}H_{26}O_5$ . The formation of this series of substances thus paralleled exactly our experience with other isogenins. The above acid,  $C_{22}H_{28}O_5$ , must be a lactol acid



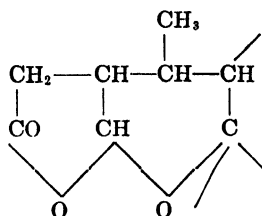
which on oxidation as the ester gives directly a lactone ester. Simultaneously, the hydroxyl group which had emerged from the above acetolysis reaction as the acetate is oxidized to carbonyl

<sup>2</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **63**, 123 (1925).

and is therefore of secondary character. This is presumably identical with  $\text{OH}^{\text{III}}$  of strophanthidin and related aglucones.

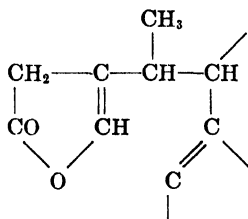
In the above formulæ we are adopting the extension of the lactone side chain in accordance with the results of our recent work on dihydrogitoxigenin.<sup>3</sup> This assumption, however, requires further confirmation.

It may be concluded, therefore, in confirmation of our previous results that ouabain is an unsaturated  $\Delta^{\beta,\gamma}$ -lactone which carries a hydroxyl ( $\text{OH}^{\text{I}}$ ) in reactive proximity to this lactone group and which is involved in the isomerization to isouabain. Thus, the configuration characteristic of the isogenin, as shown in the formula



does not participate in the above acetolysis reaction. Since this acetolysis otherwise parallels that of dihydrodesoxyouabain heptacetate, definite conclusions can be drawn as to the relationship to the isogenin of the latter substance and therefore of its parent substance, the anhydroouabain heptacetate of Arnaud.

The extra double bond of anhydroouabain heptacetate arises from the loss of the tertiary hydroxyl ( $\text{OH}^{\text{I}}$ ) of the aglucone as follows:



The excellent analytical results obtained with the new substances all conform very well with our previous conclusion that

<sup>3</sup> Jacobs, W. A., and Elderfield, R. C., *J. Biol. Chem.*, **100**, 671 (1933).

ouabain is a  $C_{26}$  compound and its genin  $C_{23}H_{34}O_8$ . Unfortunately again, however, the exact identity of the carbon atom lost as formaldehyde on acetolysis is not certain and the use of these aglucone derivatives for purposes of further structural correlation with strophanthidin is rendered difficult.

#### EXPERIMENTAL

*Acetyltrianhydrohydroxylactone*,  $C_{24}H_{28}O_6$ —To 10 gm. of dry, finely powdered isouabain, suspended in 100 cc. of acetic anhydride, were added 2 cc. of sulfuric acid. The isouabain dissolved rapidly, and the temperature of the reaction mixture rose to about  $60^\circ$ . The solution was kept at  $70^\circ$  for 15 minutes and then poured, with stirring, into 800 cc. of water containing enough sodium acetate to neutralize the sulfuric acid. The mixture was allowed to stand until the oily product became solid. The dried powder was recrystallized from ethyl alcohol. The yield was 3 gm. Two recrystallizations from ethyl acetate gave 2 gm. of stout rhombic prisms which melted at  $244\text{--}246^\circ$ .

The substance is fairly readily soluble in hot ethyl and methyl alcohols, chloroform, and acetone; but sparingly soluble in cold methyl and ethyl alcohols; and almost insoluble in ether and petroleum ether. In sulfuric acid it developed a lemon-yellow color.

$$[\alpha]_D^{25} = -22.7^\circ \quad (c = 1.00 \text{ in pyridine})$$

For analysis it was necessary to dry the substance at  $120^\circ$  and 15 mm.

$C_{24}H_{28}O_6$ .	Calculated.	C 72.69, H 7.12
	Found.	" 72.63, " 7.11

13.044 mg. of substance were refluxed for 4.5 hours in 2 cc. of alcohol and 3.5 cc. of 0.1 N NaOH. Calculated for 2 equivalents, 0.659 cc.; found, 0.690 cc.

The aqueous mother liquor from the above crude acetolysis product was distilled slowly into ice water. The distillate gave a strong resorcinol ring test. 150 cc. of the distillate, after treatment with an excess of ammonia, were allowed to stand overnight. After concentration it was treated with an alcoholic solution of iodine. 30 mg. of hexamethylenetetramine tetraiodide, which was identified by the melting point, were obtained.

*Hydroxytrianhydrolactone*,  $C_{22}H_{26}O_4$ —0.1 gm. of the acetyl-trianhydrolactone was boiled for a half hour with an excess of 0.5 N sodium hydroxide in 50 per cent alcoholic solution. The reaction mixture was then cooled and made acid to Congo red paper with hydrochloric acid. Ethyl alcohol was then added until the resinous precipitate completely redissolved. After standing 2 days at room temperature, 80 mg. of rectangular platelets had separated out. After two recrystallizations from a large volume of boiling ethyl alcohol, the compound melted at 256–258°. It was sparingly soluble in hot methyl and ethyl alcohols and in chloroform, very sparingly soluble in acetone and ethyl acetate, and practically insoluble in ether.

$$[\alpha]_D^{22} = +29.1^\circ \text{ (} c = 1.00 \text{ in pyridine)}$$

For analysis the substance was dried at 120° and 15 mm.

$C_{22}H_{26}O_4$ .	Calculated.	C 74.53, H 7.40
	Found.	" 74.50, " 7.29

The marked difference in optical activity between this substance and its previous acetate suggests that some rearrangement may have occurred during the saponification.

*Hydroxytrianhydrolactol Methyl Ester*,  $C_{23}H_{30}O_5$ —1 gm. of the acetyltrianhydrolactone was saponified in 50 per cent ethyl alcoholic solution with an excess of 0.5 N sodium hydroxide for three-quarters of an hour. The reaction mixture was then diluted with water and carefully acidified with acetic acid, an excess of the reagent being avoided. The solution was extracted three times with chloroform. All manipulations were performed as rapidly as possible because of danger of relactonization. The chloroform solution was washed with water and dried rapidly over sodium sulfate and by a short distillation under diminished pressure. It was then treated with a slight excess of diazomethane. After concentration, the addition of a small volume of alcohol yielded 0.53 gm. of thin rectangular platelets. Recrystallization from a fairly large volume of alcohol gave stout rhombs which melted at 214–216° with effervescence. The substance is fairly readily soluble in boiling methyl and ethyl alcohols, sparingly soluble in chloroform and acetone, and almost insoluble in ether. The solu-



tion in sulfuric acid is a bright orange-yellow, which changes finally to a bright vermillion.

$$[\alpha]_D^{25} = +1.6^\circ \quad (c = 0.985 \text{ in pyridine})$$

$C_{23}H_{26}O_5$ . Calculated. C 71.46, H 7.83,  $OCH_3$  8.03  
 Found. " 71.44, " 7.90, " 7.93

*Ketotrianhydrolactone Methyl Ester*,  $C_{23}H_{26}O_5$ —A suspension of 0.2 gm. of hydroxytrianhydrolactol methyl ester in 2 cc. of 90 per cent acetic acid was treated with 1 cc. of Kiliani chromic acid solution. The substance gradually dissolved. After 7 minutes the reaction mixture was diluted with 5 times its volume of water and then extracted with chloroform. The residue crystallized under methyl alcohol. The yield was 70 mg. of ester. Recrystallization from chloroform-ethyl alcohol gave rectangular plates which melted at 210–213°. The solution in sulfuric acid is a very pale green-yellow which rapidly fades.

$$[\alpha]_D^{25} = +172.5^\circ \quad (c = 1.025 \text{ in pyridine})$$

For analysis the substance was dried at 110° and 15 mm.

$C_{23}H_{26}O_5$ . Calculated. C 72.21, H 6.86,  $OCH_3$  8.12  
 Found. " 72.09, " 6.80, " 7.96

In the following saponification experiments somewhat more than the 2 equivalents of alkali required by the ester and lactone groups was consumed. This was due unquestionably to more deep-seated decomposition, since the substance is an unsaturated ketone.

13.935 mg. of substance were refluxed for 4.5 hours in 2 cc. of alcohol and 3.5 cc. of 0.1 N NaOH and titrated back against phenolphthalein. Calculated for 2 equivalents, 0.729 cc.; found, 0.866 cc.

12.920 mg. of substance after boiling 3 hours required 0.807 cc.; calculated, 0.676 cc.

# THE EFFECT OF THE INGESTION OF WATER AND OF UREA ON THE CHOLESTEROL CONTENT OF THE PLASMA\*

BY MAURICE BRUGER† AND CHARLES A. POINDEXTER†

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(Received for publication, March 24, 1933)

A recent report from this laboratory demonstrated that following the ingestion of glucose by fasting subjects the cholesterol content of the blood showed irregular variations (1). Apparently, in these experiments we were dealing with three or more distinct factors which may have influenced the level of the plasma cholesterol. The glucose (and the urea in the present study) was given dissolved in approximately 250 cc. of water and an additional 250 cc. of water were usually taken to rinse the mouth. The following experiments were carried out to determine what influences, if any, the drinking of such quantities of water or even larger amounts at one time (within a few minutes) exerted on the physiological variations of the plasma cholesterol. Again, an increment of glucose in the blood, besides exerting purely physical influences (*i.e.* increased osmotic pressure, etc.), results in certain metabolic changes (*i.e.* augmented glycogen formation and glucose oxidation, etc.). A study of the fluctuations of the cholesterol content of the plasma following the ingestion of urea may afford some evaluation of the relative effects of the latter two factors since in a large measure metabolic influences would thus be eliminated.

## *Procedure*

Each of nine fasting subjects drank 500 or 1000 cc. of tap water within 1 to 4 minutes. The plasma cholesterol was determined

\* Aided by a grant from the Josiah Macy, Jr., Foundation.

† Oliver Rea Fellow in Medicine.

immediately before and at hourly intervals for 4 hours following the water drinking. Each of twelve fasting subjects was fed urea in doses varying from 0.04 to 1.10 gm. per kilo of body weight. The urea was given dissolved in approximately 250 cc. of water; an additional 250 cc. of water were usually allowed to rinse the mouth. The total amount of fluid taken with the urea was approximately 500 cc. The cholesterol content of the plasma and the urea nitrogen of the whole blood were determined immediately before and at hourly intervals for from 4 to 6 hours after the urea (and water) ingestion. In every instance the experiment was carried out in the morning, no food or water having been taken since dinner the evening preceding the test (15 hours post-absorptive). In the four cases receiving 1000 cc. of water and occasionally following the ingestion of urea, hematocrit studies were made. The plasma cholesterol was determined by Sackett's method (2) modified by the temperature control procedure used in this laboratory (3); the urea nitrogen by the gasometric urease method of Van Slyke (4).

### *Results*

Bruger and Somach (5) have reported the hourly fluctuations of the plasma cholesterol over a period of 5 hours in seven fasting subjects without water drinking. Their data suggested that variations of the plasma cholesterol greater than  $\pm 7.8$  per cent (twice the observed standard deviation) should be considered significant changes when any aspect of cholesterol metabolism is studied over a similar period of time (5 hours). Table I demonstrates that following the ingestion of 500 or 1000 cc. of water, only one out of nine subjects (Case 6) shows a deviation greater than  $\pm 7.8$  per cent. If we assume the maximal deviation from the control as indicative of the probable results, then it appears that six subjects show a fall in the plasma cholesterol and three a rise, but it should be pointed out that in only one instance (Case 6) is the deviation from the control greater than we have observed in fasting subjects without water drinking. The hematocrit studies (Cases 6 to 9, Table I) show that a definite degree of blood dilution occurs following the ingestion of 1000 cc. of water, but in only one out of four instances (Case 6) did the plasma cholesterol decrease appreciably. In three instances no parallel relation was

observed between the cholesterol and the water content of the blood.

Table II shows the hourly variations of the cholesterol content of the plasma and the urea nitrogen of the whole blood over a period of 4 to 6 hours in twelve fasting subjects following the

TABLE I

*Hourly Variations of Plasma Cholesterol in Nine Fasting Male Subjects Following Ingestion of Water, Including Hematocrit Studies in Four Cases*

Case No.	Age <i>yr</i>	Diagnosis	Water ingested <i>cc</i>	Plasma cholesterol (mg per 100 cc) and corpuscle volume per cent (hematocrit)*					Percentage deviation of	
				Control	1 hr	2 hrs	3 hrs	4 hrs	Maximum cholesterol from control	Minimum cholesterol from control
1	36	Normal	500	207	203	205	194	219	5 8	6 2
2	48	Arteriosclerosis	500	211	207	219	211	212	3 8	1 9
3	42	Carcinoma of bronchus	500	166	164	161	154	153	0	7 8
4	54	Diabetes mellitus	500	284	284	276	286	264	0 8	7 0
5	32	Polyposis of bronchi	500	206	213	214	217	211	5 3	0
6	28	Normal	1000	226	201	192	184	186	0	18 5
				<b>39.5</b>	<b>37.8</b>	<b>36.9</b>	<b>35.4</b>	<b>37.4</b>		
7	42	Carcinoma of bronchus	1000	200	205	204	202	207	3 5	0
				<b>41.4</b>	<b>39.7</b>	<b>38.0</b>	<b>37.4</b>	<b>36.5</b>		
8	40	Migraine	1000	148	143	145	141	148	0	4 6
				<b>43.6</b>	<b>42.3</b>	<b>40.5</b>	<b>38.7</b>	<b>40.0</b>		
9	41	Arthritis	1000	177	175	168	168	168	0	5 0
				<b>46.8</b>	<b>46.1</b>	<b>45.9</b>	<b>44.7</b>	<b>45.2</b>		

\* Bold-faced figures represent hematocrit readings.

ingestion of various amounts of urea. A dose of 0.04 gm. per kilo of body weight was accompanied by little change in the urea content of the blood; with larger quantities (0.21 to 1.10 gm. per kilo of body weight) a rise always occurred. The maximal urea concentration in the blood was usually observed 2 hours following

TABLE II  
*Variations of Plasma Cholesterol and Whole Blood Urea Nitrogen Following Ingestion of Various Amounts of Urea in Twelve Fasting Subjects*

Case No	Age and sex	Body weight kg	Diagnosis	Experiment	Cholesterol per 100 cc plasma and urea N per 100 cc whole blood*							Percentage deviation of	
					Control	1 hr	2 hrs	3 hrs	4 hrs	5 hrs	6 hrs	Maximum cholesterol from control	Minimum cholesterol control
1	53 M.	56 4	Gastric neurosis	0 04 gm. urea per kilo body weight	mg 189	mg 195	mg 178	mg 187	mg 177			3 1	6 3
2	42 "	79 1	Arteriosclerosis	"	9.3	10.6	10.7	10.7	11.3			0	5 0
3	63 "	60 4	"	0 21 gm. urea per kilo body weight	200	191	194	190	192			2 4	10 4
4	52 "	100 0	Arteriosclerosis	0 65 gm. urea per kilo body weight	16.9	15.9	16.8	16.4	14.2			0	24 8
5	15 "	56 3	Hypertension	"	286	286	259		293	16.9		0 8	6 5
6	54 "	49 5	Arteriosclerosis	"	7.7	21.4		410		335		0	18 5
7	34 "	82 0	Chronic arthritis	"	446	399		49.4		48.0		34.2	7 1
8	62 "	75 0	Arteriosclerosis hypertension	"	366	369	342		352	368		14 6	21 3
					7.6	38.7	43.8		38.8	34.3	269	0	
					330	309	282		282		34.2	329	
					12.9	40.9	45.6		38.9		36.3	36.3	
					334	310	317		383		281	0	
					7.5	30.4	44.5		41.1		351		
					357	284			351		39.9		
					14.9		46.8		43.3				

9	36 M.	62 7	Normal	0.65 gm. urea per kilo body weight	189	161	163	176	176	176	176	0	14 8
10	50 F.	54 5	Hypertension	"	<b>12.6</b>	<b>39.8</b>	<b>43.7</b>	<b>42.6</b>	<b>37.6</b>	<b>33.7</b>		0	23.4
11	67 "	56 7	Arteriosclerosis	1.10 gm. urea per kilo body weight	426	408	379	340	350	326		0	
					14.8	52.8	70.7	65.2	65.3	62.1			
12	57 M	63 2	Thromboangiitis obliterans	"	350	315		308			364	4 0	12 0
					<b>14.6</b>	<b>84.7</b>	<b>70.8</b>				<b>72.8</b>	6 5	11 9
					277	281	250	244	280	295			
					<b>12.5</b>	<b>53.0</b>	<b>74.6</b>	<b>76.7</b>	<b>67.3</b>	<b>63.6</b>			

\* Bold-faced figures represent urea nitrogen values.

the ingestion of urea; at the 5th or 6th hour it was still markedly elevated, especially after the administration of the larger doses.

In Cases 1 and 2 (Table II) the absence of any change in the urea nitrogen content of the blood was associated with no striking deviations of the plasma cholesterol; the variations were no greater than were observed in the subjects drinking 500 cc. of water (Cases 1 to 5, Table I) or in fasting subjects without water drinking (5). The increase in the urea nitrogen in Cases 3 to 12 inclusive was accompanied by a distinct fall in the cholesterol in eight instances (Cases 3, 4, 6, 8 to 12), a definite rise in one (Case 7), and practically no significant alteration in another (Case 5). No correlation, however, was observed between the degree of rise in the urea nitrogen of the blood and the extent of the fall of the plasma cholesterol. Thus Cases 11 and 12 showed a greater increase in the urea nitrogen than did Cases 4, 6, 8, and 10 (because of the larger amount of urea ingested); nevertheless, the decrease of the plasma cholesterol was not as marked as in the latter group. In four of the eight cases showing a distinct fall of the cholesterol the prolonged rise of the urea nitrogen was accompanied by a persistent diminution of the plasma cholesterol (Cases 4, 6, 8, 10); in the remaining four the cholesterol decreased markedly but subsequently rose while the urea nitrogen was still elevated (Cases 3, 9, 11, 12).

#### DISCUSSION

Heilig and Lederer (6) studied the cholesterol content of the blood in several pathological cases before and after the diuresis produced by various drugs. They reported the unexpected finding that in cases with cardiac or amyloid disease hydremia is associated with an increased cholesterol content of the blood and anhydremia with a decreased cholesterol. These results and ours, though not strictly comparable, are in apparent disagreement; however, we would conclude that the degree of hydremia is not the only factor concerned in modifying the level of the plasma cholesterol, that the method employed to increase or decrease the water content of the blood must also be considered as having a possible influence on the fluctuations of the cholesterol content of the plasma.

Very recently Okey and Stewart (7) have reported the effect of diet on the blood cholesterol and suggested that the diurnal varia-

tions of the blood cholesterol noted by Bruger and Somach (5) may have been influenced by water ingestion. The results reported in this communication show that this factor is probably negligible.

The protocols demonstrate that the ingestion of urea is usually accompanied by a distinct fall of the plasma cholesterol only when the urea nitrogen of the blood is elevated significantly. Peters and Van Slyke (8) state that following the administration of urea a transient blood dilution and a subsequent blood concentration occur. In a few instances when hematocrit studies were made following the ingestion of urea, we observed a tendency to a slight blood concentration in all samples, but since our first experimental blood was drawn 1 hour after the administration of urea, it is possible that we missed the period of hydremia. It follows, therefore, that the decrease of the plasma cholesterol, which persisted at times for 6 hours, cannot be accounted for by the observed fluctuations of the water content of the blood.

In view of our present knowledge of urea, as an end-product of protein catabolism, it seems unlikely that the ingestion of this substance is associated with any metabolic phenomena which may be responsible for the observed changes in the blood cholesterol. We are of the opinion that an increase in the urea content of the blood is accompanied by a rearrangement of other blood constituents, probably as an attempt at osmotic equilibration. It is still a moot question whether cholesterol, *per se*, exerts any appreciable osmotic effect; the changes in the plasma cholesterol, at least, may indicate that an increment in the blood urea modifies the plasma colloid structure in such a way that the cholesterol tends to decrease appreciably.

Simultaneous studies of the plasma cholesterol and the blood urea in many cases of chronic nephritis extending over a period of about 2 years (results to be reported) indicate an inverse relation between cholesterol and urea in many instances. A number of observers have recorded the low plasma cholesterol and the marked nitrogen retention in the uremia of chronic nephritis (9). Apparently, an increase in the blood urea, whether due to the renal insufficiency of chronic nephritis or to urea ingestion in subjects with normal kidneys, is often accompanied by a diminution of the plasma cholesterol.

Glucose resembles urea in many of its physical properties and



it would thus appear that a rise in the glucose concentration of the blood would result in a fall of the cholesterol content of the plasma. The additional influences of metabolic changes, however, probably account for the variable results so frequently observed following the ingestion of glucose.

#### SUMMARY

In each of five fasting subjects drinking 500 cc. of water the variations of the plasma cholesterol were no greater than were observed in the control cases without water drinking. In each of four subjects drinking 1000 cc. of water a definite fall in the hematocrit was observed, but in only one instance did the cholesterol decrease appreciably.

In twelve fasting subjects fed various doses of urea an increase in the urea concentration of the blood was usually associated with a decrease of the cholesterol content of the plasma. Fluctuations in the water content of the blood did not account for the observed variations in the plasma cholesterol. The assumption is made that an increment in the blood urea modifies the plasma colloid structure in such a way that the cholesterol tends to decrease appreciably.

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## LIVER INJURY AND BLOOD LACTIC ACID\*

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(Received for publication, March 13, 1933)

Normal and abnormal liver function has been of interest to workers (13) in this laboratory for some time. Any test related to the internal metabolism of the liver which might put an extra burden on this organ would prove of interest to the physiologist and perhaps of use to the physician. It is well known that the functional reserve of the liver has a large margin of safety.

It is generally believed that the muscles are the main source of lactic acid in the body and the liver is largely responsible for its removal from the blood (9). It was decided to inject intravenously considerable amounts of sodium lactate or lactic acid and follow the curve of disappearance from the blood. If the liver is largely responsible for this removal of lactic acid there should be a change in the speed of removal of lactic acid from the blood when the liver is seriously injured.

Space will not permit a review of the literature (1, 3, 4, 10, 11) but papers have been consulted which relate to this question. Bollman and Mann (2) have recently reported that complete extirpation of the liver does not prevent the disappearance of lactic acid from the blood following intravenous injection.

### *Methods*

Large, adult dogs (16 to 22 kilos) on a kennel ration of hospital scraps were used for the experiments. They were kept as quiet as possible for at least a half hour before each experiment.

Control level samples were first obtained from the neck veins. All oxalated blood samples were chilled, poisoned with sodium

\* The writer wishes to express his appreciation of the advice and assistance of Drs. G. H. Whipple, W. B. Hawkins, and F. S. Daft.

fluoride, or treated to remove proteins as soon as possible to prevent glycolysis.

The dogs were then injected by means of the jugular or external saphenous veins with 100 ml. of lactic acid solution (10 ml. of concentrated lactic acid, sp. gr. 1.20, diluted to 100 ml.) or with 100 ml. of sodium lactate solution (10 ml. of concentrated lactic acid, sp. gr. 1.20, diluted with about 15 ml. of water and then neutralized with sodium hydroxide to a point where it had an acid reaction to phenol red while just alkaline to litmus, the whole then diluted to 100 ml.).

The time of injection varied from 2 to 12 minutes depending on the clinical reaction of the animal.

Immediately after injection samples were taken at intervals of 10 or 15 minutes but after the 1st hour the intervals were increased gradually to an hour.

The animal after a few days interval was then fasted for 2 days, subjected to 1 hour of light chloroform anesthesia, and 48 hours later again injected and samples taken. In the case of one animal a recovery period of 10 days was followed by another injection.

Proteins were precipitated from 10 ml. of oxalated blood samples by the Folin-Wu (6) tungstic acid procedure and carbohydrates were subsequently removed from the protein filtrates by the method of Van Slyke (12). Aliquot portions of the centrifugate, or the filtrate if filtered, were then analyzed for lactic acid by a modification of the Friedemann, Cotonio, and Shaffer method (7). Oxidation was accomplished with colloidal manganese dioxide according to Friedemann and Kendall (8). Absorption of the aldehyde by the modification of Davenport and Davenport (5) with 3 to 4 ml. of bisulfite was found to be successful except that in using large test-tubes as receivers in the place of flasks difficulties were encountered in titration. The bisulfite was therefore washed into flasks at the end of the oxidation procedure.

0.1 N and 0.002 N iodine were employed in titration and the bound bisulfite was liberated by means of a pinch of sodium bicarbonate.

#### *Experimental Observations*

Dog 30-224, a 22 kilo, female, adult, mongrel pointer was injected with lactic acid under normal conditions on three occasions.

This dog had previously had a closed fistula operation but later the common duct had become reestablished and the dog was normal. The control levels in the three experiments were 36, 20, and 8 mg. per cent of blood lactic acid and all curves showed a similar rate of disappearance of blood lactic acid regardless of these widely varying control levels (Chart 1). The first liver injury following 2 days fast was accomplished by 1 hour of light chloroform anesthesia. 2 days later the dog was again injected and the resulting curve was essentially the same as the others. At this time the dog showed jaundice in the eyeballs and the blood clotted slowly. A month later the same dog was subjected to  $1\frac{1}{4}$  hours of

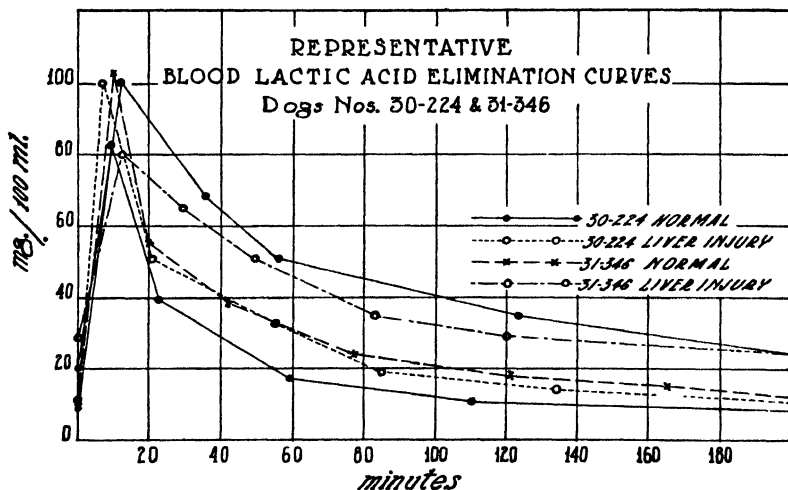


CHART 1

light chloroform anesthesia following a 2 day fast and again injected with lactic acid, this time 5 ml. of 1:20,000 adrenalin being incorporated with the injection solution with the idea of inhibiting the formation of muscle glycogen from blood lactic acid as well as the extent of the oxidation of lactic acid, according to the concept of Cori. At this time the jaundice was very marked, being noticeable in the mucous membranes of the mouth, the eyeballs, the skin over the groin, and plasma. Blood clotted after much delay. The response to injection was much less marked by hyperpnea than in the previous experiments without adrenalin and the pulse was strong throughout the experiment. Multiple hematoma made

blood sampling difficult and samples of sufficient amount for analysis were obtained only at scattered intervals. The dog died subsequent to the experiment of hemorrhage and delayed chloroform poisoning. Analysis of representative samples showed the same immediate fall in blood lactic acid as observed before.

Autopsy of the dog showed no changes of interest for these experiments except in the liver which was grossly very yellow. The larger bile ducts were somewhat dilated and bile flowed into the duodenum. Histologically the liver showed some increase of stroma and new bile ducts in the portal areas. The central two-thirds of each liver lobule was abnormal and contained liver cells which were necrotic or filled with fat droplets. This liver injury was in large part responsible for the bleeding which was the immediate cause of death. The portal reaction was related to the old history of a biliary fistula and partial obstruction.

Dog 32-59, a normal male mongrel pointer, 12 kilos in weight, was injected with lactic acid solution and the resulting curve was similar to the normal curves of the preceding dog.

Dog 32-87, a normal female mongrel police dog, showed a similar curve on injection of lactic acid solution.

Dog 31-346, a normal female police dog, 17 kilos in weight, was given injections of racemic sodium lactate on two occasions under normal conditions. The curves of the blood lactic acid were nearly identical and of the usual type. A 2 day fast was followed by 1 hour of light chloroform anesthesia and another injection after 48 hours. At this time there was very little clinical evidence of liver damage as the blood clotted easily (4 minutes) and the plasma showed no jaundice. The blood lactic acid curve was very much like the normal.

6 days later the dog was fasted for 2 days and chloroformed for 1 hour. After 48 hours another injection was made. At this time the plasma was definitely icteric and although the clotting time of the blood was 4 minutes the clot was soft. The lactic acid curve was similar to the others (Chart 1).

Following a 10 day period of liver regeneration, the plasma having again become colorless 2 days after the injection, another sodium lactate injection was made and the resultant curve was quite like the other normal curves.

## DISCUSSION

The elimination curves of lactic acid following hepatic injury are essentially the same as the normal control curves. Since all curves are within normal limits, it is quite clear that this procedure is of no value for the determination of liver function. The results would seem to indicate either that the margin of safety of the liver is so great that severe injury of liver cells causes no disturbance of glycogenic function, or that in the event of the liver becoming functionally incapacitated other tissues (probably muscles, for the most part) which are associated with the fixation or utilization of lactic acid take over the added load. That the latter is the most probable explanation is indicated by the recent work of Bollman and Mann (2) in which it was reported that hepatectomy did not prevent the disappearance of lactic acid from the blood following intravenous injection.

The diluted lactic acid, when injected as such, has a profound effect on the vascular system resulting in marked hyperpnea and a fall in blood pressure. The veins usually become thrombosed at the point of injection and there was noted, in all cases where excretion took place, a definite hematuria. If the material is injected too rapidly, fatal termination may result. About 2 ml. per kilo per minute or slightly less of the solution of this strength is a fairly safe rate. Clinical recovery following the lactic acid injection is quite rapid, only one dog (chloroform injury) showing any serious delayed reaction, death in this case resulting from pulmonary edema 2 hours after the lactic acid injection. The injection of sodium lactate did not give rise to any untoward clinical symptoms regardless of the injection rate.

## SUMMARY

When considerable amounts of lactic acid or sodium lactate are given intravenously in normal dogs there is a rapid disappearance of this substance from the blood stream.

When the liver has been severely injured by chloroform poisoning the disappearance curve for lactate in the blood is like that in the normal dog.

If the liver is normally concerned with the removal of lactic acid from the blood, some emergency alternative reaction comes into play when the liver is functionally disturbed or incapacitated.

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## THE OXIDATION OF CYSTEINE WITH IODINE: FORMATION OF A SULFINIC ACID\*

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The mechanism of the reaction involved in the oxidation of the sulfhydryl group to the disulfide form has been studied by many investigators. The results reported in this paper indicate the intermediate steps in the oxidation of cysteine to cysteic acid.

Bierich and Kalle (2) made a study of the iodine titration of cysteine and found that tissue extracts gave too high values for their  $-SH$  content with the starch-iodide method of Okuda (7) and with the nitroprusside method of Tunncliffe (10). The same held true for pure cysteine and reduced glutathione. They found that the dilution factor was of very great importance for the values were higher the greater the dilution. Okuda (7) stated the possibility of two reactions going on in competition with one another, that is oxidation of cysteine to cystine or to cysteic acid. By proper conditions Okuda suppressed the latter reaction. Since Bierich and Kalle (2) obtained a dilution factor up to 100 per cent, they assumed that both reactions took place. If increasing amounts of potassium iodide were added, formation of cysteic acid could be suppressed practically to zero.

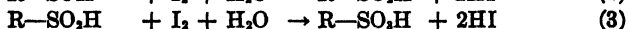
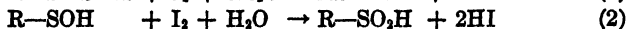
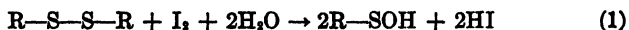
The work of many other investigators also shows that the iodometric titration of sulfhydryl compounds may give variable results and suggests the possibility of a series of steps which involve gradual addition of oxygen in the building up of cysteic acid which is the end-product. That cysteine may be oxidized

\* This paper is an abridgment of a thesis submitted to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry.

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quantitatively to cysteic acid by means of iodine has been shown by Shinohara (9) who isolated the theoretic amount of cysteic acid. Later he (9) proposed the following mechanism for this reaction.



Dowler (3) found that cysteine was not oxidized quantitatively to cystine, provided the cysteine was added to the iodine solution. A further study of this reaction has been carried out in the hope that some intermediate compound could be isolated as a chemical entity.

In the reinvestigation of this problem, the effect of dilution and acidity was first studied. Contrary to Okuda's results variations in acidity produced no difference in the amount of iodine consumed by the cysteine. The dilution factor was the important one for variations of from 100 to over 200 per cent were observed. Table I shows the effect of the addition of potassium iodide on the iodine consumption of cysteine when the cysteine was added to the iodine. With greater dilution with acid more iodine was consumed by the cysteine, thus showing the effect of dilution as well as iodide.

A series of experiments was performed in which 0.01 N cysteine was added to 0.01 N iodine solution. No potassium iodide other than that already in the solution of iodine was added (0.1 N iodine was prepared, 25 gm. of potassium iodide being used per liter to effect solution, and this was diluted to 0.01 N for titration). Starch was used as an indicator. When the titrations were completed the cystine content of the solutions was determined by a modification of the Folin-Looney method (4). The iodine consumed was far in excess of that necessary to convert the cysteine to cystine. When the amount of iodine required for the oxidation of the cysteine to the cystine, which was determined to be present, was subtracted from the total amount consumed, it was found that 4 equivalents had been used by each molecule of cysteine. The results in Table II offer evidence that some of the cysteine had been oxidized to a compound intermediate between cystine and cysteic acid, possibly to a sulfinic acid.

TABLE I

*Effect of KI and Absence of KI on Iodine Consumption of Cysteine\**

10 cc. 0.00947 N iodine in 2.5 per cent HCl, 5 cc. 5 per cent KI, 1 cc. 0.5 per cent starch		10 cc. 0.00998 N iodine in 2.5 per cent HCl, no KI, 1 cc. 0.5 per cent starch	
Dilution with 2.5 per cent HCl	0.01 N cysteine consumed	Dilution with 2.5 per cent HCl	0.01 N cysteine consumed
cc.	cc.	cc.	cc.
0	11.00	0	4.92
10	7.54	10	3.75
20	6.61	20	3.68
30	5.37	30	3.47
40	4.92	40	3.30
50	4.18	50	3.22
60	3.62	60	3.18
70	3.41	70	3.04
80	3.46	80	2.93
90	3.42	90	2.65
100	3.34	100	2.54

\* Cysteine was added to the iodine solution.

TABLE II

*Equivalents of Iodine Reduced by Cysteine in Oxidation beyond Disulfide Stage\**

10 cc. samples of 0.00996 N iodine were used.

Dilution with 2.5 per cent HCl	0.00993 N cysteine consumed	Cystine recovered	Iodine necessary for cystine formed	Calculation of equivalents of iodine reduced by cysteine oxidised beyond cystine
cc.	cc.	mg.	cc.	
0	4.45	2.86	2.67	4.10
10	3.58	1.61	1.34	3.87
20	3.30	1.50	1.25	4.36
30	3.25	0.86	0.72	3.68
40	3.17	0.79	0.65	3.70
50	3.15	0.84	0.70	3.79
60	3.10	0.57	0.48	3.64
70	3.00	0.51	0.43	3.73
80	2.96	0.39	0.31	3.66
90	2.97	0.47	0.41	3.78
100	2.95	0.58	0.48	3.86

\* Cysteine was added to the iodine; 1 cc. of 0.5 per cent starch as indicator in control.

Proof that a sulfinic acid had been formed was made in the following manner. If no cystine or cysteine was present, the sulfinic acid should reduce 2 more equivalents of iodine and form cysteic acid. If any cysteic acid was present less than 2 equivalents of iodine would be reduced. A series of experiments was performed in which several samples of the sulfinic acid (slightly less than 4 mg. in each sample) were prepared and then treated with an excess of iodine. The results in Table III show that 2 more equivalents of iodine are reduced by the sulfinic acid. This reaction proceeds more quickly as the temperature is increased.

TABLE III

*Effect of Time and Temperature on Oxidation of Sulfinic Acid with Iodine*

Equiva- lents of iodine on first titration	Temper- ature	Iodine after an excess has been added							
		Hrs.	Equiva- lents	Hrs.	Equiva- lents	Hrs.	Equiva- lents	Hrs.	Equiva- lents
	°C.								
4 13	22-24	24	5 23	48	5 37	72	5 52	120	5.55
4 17	35-38	24	5 38	48	5 57	96	5 74	120	5.70
4 17	50-52	24	5.50	48	5 62	76	5 75	92	5.84
4 16	60	24	5 80	48	5 93	72	6 05	120	6.03
4 16	70	24	5 94	48	6 04				

Great difficulties were encountered in the isolation of the sulfinic acid. Hydriodic acid was used to effect solution of iodine as potassium iodide was difficult to remove. In the first experiments the hydriodic acid was not removed but with increasing amounts of the sulfinic acid reduction of this compound by the hydriodic acid occurred readily even in the cold. This reduction was subsequently prevented by immediate removal of the hydriodic acid with silver oxide. When sulfuric acid was used in place of hydrochloric acid, removal of the sulfuric acid resulted in almost complete loss of the sulfinic acid. Barium hydroxide, lead acetate, and sodium carbonate with subsequent precipitation of the sodium sulfate with alcohol were used to remove the sulfuric acid but the precipitates adsorbed the larger part of the sulfinic acid.

A fraction containing a product which had the properties of a

sulfinic acid was finally separated as described in the experimental procedure.

#### EXPERIMENTAL

500 cc. of 0.01 N iodine, made by dilution of 0.1 N iodine which contained 15 to 17 cc. of 57 per cent hydriodic acid in each liter, were placed in a 5 liter flask and 300 cc. of 10 per cent hydrochloric acid and 2200 cc. of water were added. This made the concentration of hydrochloric acid 1 per cent. The solution was stirred rapidly with a mechanical glass stirrer while 0.5 N cysteine hydrochloride was added. A control, containing starch, showed that from 2.48 to 2.53 cc. of cysteine hydrochloride were necessary; therefore, to each flask containing the above amount of iodine, 2.5 cc. of cysteine were added. The latter was added from a 1 cc. burette; the time for addition of the cysteine was less than 2 minutes. A decided temperature factor was noticed. If the temperature varied from 22–24° the solution became colorless within 3 to 10 minutes after the addition of the cysteine. Several of these samples were prepared during the summer when the temperature was as high as 33–34° and on these days the color disappeared as soon as the last drop of cysteine was added. Under the conditions which were used, slightly less than 200 mg. of the sulfinic acid were prepared in each flask. As soon as the iodine color had disappeared 3.5 gm. of silver oxide were added and the solution was stirred rapidly for several minutes. The silver iodide which formed was removed by filtration and the filtrates were concentrated to a volume of from 2 to 5 cc. in a vacuum. Small amounts of silver oxide were added at frequent intervals during evaporation to insure complete removal of the hydriodic acid. The last traces of hydrochloric acid were removed with silver oxide or silver acetate. After removal of the silver chloride by filtration the solution was treated with hydrogen sulfide to remove any traces of silver. The filtrate after removal of the silver sulfide was concentrated in a vacuum to a volume of 1 to 2 cc. and then poured into a 50 cc. centrifuge tube which contained absolute alcohol. After centrifuging for several minutes the mother liquor was decanted and more alcohol was added to the precipitate. The material was stirred up well with the alco-

hol and again centrifuged. After several of these treatments the precipitate became granular. The centrifuge tube was then placed in a desiccator containing sulfuric acid and a vacuum was applied until the precipitate was dry. The mother liquor was evaporated to a small volume and treated as above. Small amounts of the sulfinic acid could be recovered from the alcohol.

Table IV contains the analyses of the material isolated. Silver oxide was used to remove the hydriodic acid from Samples 1 to 4 inclusive, and silver acetate was used in Samples 5 and 6. Each sample was analyzed for cystine, cysteine (none found), nitrogen, and one was analyzed for its sulfur content.

TABLE IV

*Analysis of Fraction Containing Compound Which Possessed Properties of a Sulfinic Acid*

Sample No.	Weight	Cystine found	Nitrogen
	<i>mg.</i>	<i>per cent</i>	<i>per cent</i>
Calculated for $C_2H_7O_4NS$			9.14
1	250	None	7.34
2	520	"	7.27
3	180	"	7.65
4	250	Trace	8.09
5	600	None	7.75
6	200	12.5	7.86

Nitrogen was estimated by the Koch-McMeekin micro-Kjeldahl method (6) and the sulfur by the Benedict method (1).

Samples 3 and 4 were recrystallized as follows: The precipitates were combined, dissolved in the least possible amount of water, and the slight amount of insoluble material removed by centrifugation. Alcohol was added and the precipitate obtained gave the following analysis.

	Cystine	Cysteine	Nitrogen	Sulfur
			<i>per cent</i>	<i>per cent</i>
Found.....	None	None	7.78	18.20
Calculated for $C_2H_7O_4NS$ .....			9.14	20.93

Calculated from these values, 87.2 per cent of the recrystallized material can be accounted for as a mixture of the sulfinic acid and cysteic acid. The nitrogen and sulfur values are in good agreement.

The bromine reduced by these preparations was from 25 to 64 per cent of the theoretical amount.

Kendall (5) had observed that when cysteine was oxidized with potassium iodate at a pH of 7.8 the cysteine was not converted quantitatively to cystine. It was felt that the conditions were mild enough so that a further investigation of them might give evidence of the production of a compound intermediate between a disulfide and cysteic acid. Samples of cysteine buffered to a pH of 7.8 with phosphate buffer were titrated with potassium iodate. If potassium iodide was present the cysteine was converted almost quantitatively to cystine; if potassium iodide was absent only half of the cysteine was oxidized to cystine and the other half oxidized to a compound that reduced slightly more than 2 equivalents of iodate. This pointed to the possibility of the formation of a sulfenic acid ( $\text{RSOH}$ ) as the first intermediate produced when cysteine is oxidized with iodate.

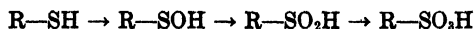
The separation of a fraction containing a compound which possessed the properties of a sulfinic acid is evidence that the mechanism of Shinohara (8) is the correct explanation of the step by step oxidation of cysteine. This is further confirmed by the evidence of the formation of a sulfenic acid. The oxidation of cysteine with iodine therefore occurs in a series of steps which involve gradual addition of oxygen in the building up of cysteic acid which is the end-product.

#### SUMMARY

A reinvestigation of the iodine titration of cysteine was made. A reversal of the usual procedure by addition of the cysteine to the iodine resulted in the formation and isolation of a fraction containing a compound that possessed the properties of a sulfinic acid. Evidence was also obtained pointing to the formation of a sulfenic acid when cysteine is oxidized with iodate.

The oxidation of cysteine occurs in a series of steps which involve gradual addition of oxygen in the building up of cysteic acid which

is the end-product. The above is offered as evidence that the following series of compounds, as suggested by Shinohara (8), are formed when cysteine is oxidized with iodine.



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# THE EFFECT OF LIGHT UPON THE VITAMIN A ACTIVITY AND THE CAROTENOID CONTENT OF FRUITS\*

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Results of recent work on the relationship of vitamin A to carotene present evidence that carotene is the plant source of vitamin A of the animal tissues. An excellent review of the recent literature pertaining to this work has appeared in the "Annual review of biochemistry" (1). The present experiments, initiated in 1928, are confined to a study of the relationship between vitamin A and the petroleum ether-soluble carotenoids of certain fruits ripened under various conditions.

The following kinds and varieties of fruits were selected for their differences in pigment content: Elberta, a deep, yellow-fleshed peach; Mayflower, a white-fleshed peach; Humboldt, a yellow-fleshed nectarine, Stanwyck, a white-fleshed nectarine; and Royal, a deep, yellow-orange-fleshed apricot. The five kinds of tomatoes selected were: Clark's Albino with pale yellow flesh; Ruby Gold with deep yellow flesh; Globe with pink flesh; California Earliana with red flesh; Gigante Ingrenole with deep red flesh. The tree fruits were allowed to ripen on the tree, (a) exposed to normal light conditions and (b) excluded from all light from the blossom stage to maturity by covering the blossom with a heavy black paper bag enclosed in white manila paper. In addition to the above treatments tomato fruits in the green mature stage were ripened by exposure to ethylene (1:2000) (12). The vitamin A and carotenoid content of greenhouse-grown and outdoor-grown tomatoes

\* These investigations form the basis of a portion of a thesis submitted by Laura Lee W. Smith to the Graduate School, University of California, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.



were also compared. A more detailed description of the method of handling the fruits is given in a previous paper by Smith and Smith (14). No chlorophyll developed in any of the fruits which were ripened from the blossom stage to maturity with all light excluded. These fruits are designated "bagged fruits" in this paper to distinguish them from those ripened under normal light conditions.

The fruit was ground and stored in small bottles having rubber stoppers equipped with inlet and outlet tubes by means of which the air was removed from the fruit and replaced by carbon dioxide. The glass tubes were then sealed and the bottles stored in the dark at  $-17^{\circ}$  until the fruit was used. These precautions were taken to insure the maximum retention of both carotenoids and vitamin A during the storage period.

As only relatively small quantities of the fruits were available for analyses, a method of pigment extraction with pyridine from wet samples was devised. This method is described in the previous paper (14). The carotenoid pigments obtained in a petroleum ether solution were determined colorimetrically with a Duboscq colorimeter and the carotene standards of Sprague (15) and Willstätter and Stoll (18). Carotene and lycopene in the petroleum ether solution were not separated. Xanthophyll was removed by treating the petroleum ether solution with 80 per cent methyl alcohol but traces of this pigment were found only in the green fruits.

The biological test for vitamin A was carried out following the procedure used in this laboratory (12). Vitamin A-depleted animals were fed the fruit for an 8 week period and an attempt was made to determine the dosage which allowed a 6 to 8 gm. weekly gain in weight. Four to ten animals were used for each dosage tested, the total number of animals being close to 400.

*Effect of Light upon Carotene Content of Tree Fruits*—Fig. 1 representing the gain in weight of the animals on equivalent doses of the different tree fruits, shows the decided superiority of the yellow over the white fruits in vitamin A content. In the yellow Elberta peach the absence of light appears to favor the development or maintenance of the carotenoid precursors of vitamin A as those fruits grown in the dark have the higher carotenoid and vitamin A content. This was not true of the other varieties of tree fruits grown. The fruits of the white and yellow nectarines, yellow apri-

cots, and the white peaches grown in the light were richer in precursors of vitamin A and carotenoid content than those grown in the dark. These results indicate that in some cases the presence of light may not aid in the production or maintenance of precursors of the vitamin in the fruits. The flesh of the white nectarines, though apparently lacking in carotenoid pigments, was not wholly devoid of vitamin A activity.

The vitamin A value of the Elberta peach compares favorably with that of the Muir peach which was reported by Morgan and Field (10) to produce a 41 gm. gain in vitamin A-free rats in 8

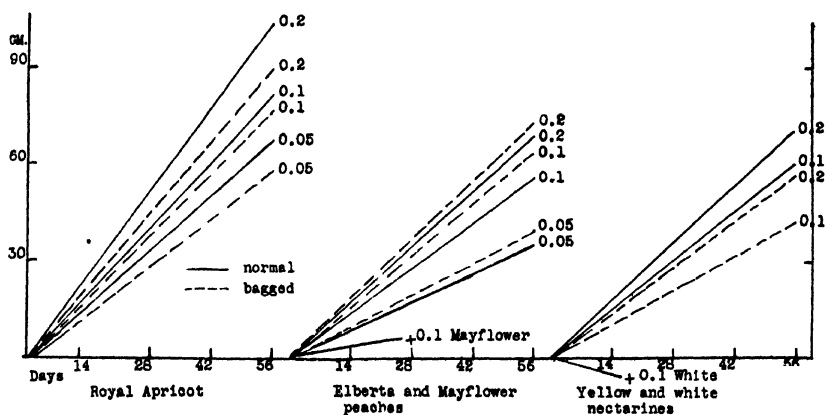


FIG. 1. Rate of growth of vitamin A-free rats fed apricots, yellow and white peaches, and yellow and white nectarines, tree-ripened and bagged. The daily dosage in gm. is written opposite the weight lines. Only one line each is given for the white peaches and nectarines.

weeks when 0.1 gm. was fed daily, the Elberta yielding 56 and 69 gm. gains. The Muir peach is also distinctly paler in color than the Elberta. The remarkably large vitamin A value of the apricot is again noted, the normally tree-ripened samples showing growth-promoting properties approached only by the normally vine-ripened Ruby Gold and Gigante Ingrengnole varieties of tomatoes.

*Relation of Level of Carotene Intake to Total Growth Secured*—As may be seen in Table I, the total gain in weight of the rats fed these fruits as sole source of vitamin A bears a certain relationship to the amount of carotenoid pigment in the daily dose and to the gains produced by similar intakes of crystalline carotene. A more ex-

TABLE I

*Relation of Total Carotene Intake in Tree Fruits and Level of Daily Dosage to Gain in Weight of Vitamin A-Free Rats*

Fruit	Carotene content	Daily intake		Survival period	Average total gain	Average weekly gain	Gain per 0.001 mg carotene	
		Fruit	Carotene				Found	Calculated
	<i>mg per kg</i>	<i>mg</i>	<i>mg</i>	<i>days</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
Royal apricot								
Tree-ripened	21.7*	50	0.0011	56	67	8.4	1.08	0.96
		100	0.0022	56	82	10.2	0.68	0.75
		200	0.0044	56	103	12.9	0.42	0.40
Bagged	8.4	50	0.0004	56	58	7.2	2.47	
		100	0.0008	56	78	9.7	1.66	
		200	0.0017	56	89	11.1	0.94	0.84
Elberta peach								
Tree-ripened	7.6	50	0.0004	56	36	4.0	1.69	
		100	0.0008	56	56	7.0	1.32	
		200	0.0015	56	70	8.7	0.82	0.84
Bagged	9.6	50	0.0005	56	40	5.0	1.49	
		100	0.0009	56	69	8.6	1.28	
		200	0.0019	56	73	9.1	0.68	0.80
Humboldt yellow nectarine								
Tree-ripened	7.2	100	0.0007	56	60	7.5	1.46	
		200	0.0014	56	67	8.4	0.83	0.90
Bagged	5.9	100	0.0006	49	41	6.9	1.42	
		200	0.0012	56	56	7.0	0.84	0.94
Crystalline carotene, m.p. 180°			0.0010	56	54	6.9	0.98	
			0.0020	56	87	10.9	0.78	
			0.0050	56	104	13.0	0.37	
Mayflower white peach								
Tree-ripened		0.100 to		16 to	-4 to			
		0.500		41	+13			
Bagged		0.100 to		12 to	-3 to			
		0.500		35	+3			
Stanwyck white nectarine								
Tree-ripened		0.300 to		40 to	10 to			
		0.400		48	9			
Bagged		0.300 to		16 to	-2 to			
		0.400		30	-1			

\* Similar tests for Royal apricots of the 1930 and 1931 crops showed 18 and 23 mg. of carotene per kilo of fresh fruit.

tended study of this relation has been reported for apricots, fresh and dried (11). It is interesting to observe that as the level of carotene fed is lowered the gain per unit of carotene increases. Thus daily doses containing less than 0.001 mg. of carotene are seen to produce gains of 1.28 to 2.47 gm. in the 56 day period per 0.001 mg. of total carotene intake, whereas 0.001 to 0.002 mg. doses produce gains of only 0.68 to 1.10 gm. and doses of 0.004 and 0.005 mg. only 0.42 and 0.37 gm. The relation appears to approximate a logarithmic curve as shown in Fig. 2.

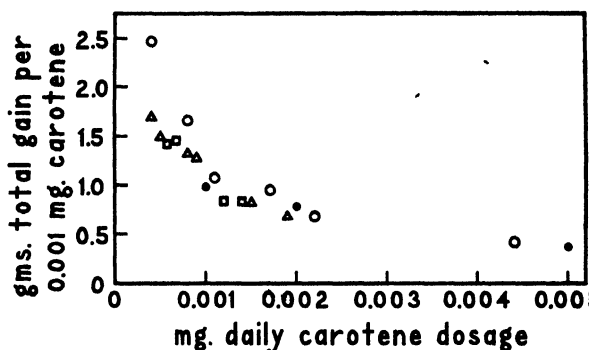


FIG. 2. Relation between daily intake of carotene and growth of vitamin A-free rats per 0.001 mg. of total carotene intake in 56 days. Solid black dots represent crystalline carotene; circles represent apricots; triangles represent Elberta peaches; squares represent yellow nectarines.

The calculated figures shown in Table I, interpolated from the crystallized carotene data, agree fairly well with the experimental data. Since no figures obtained under comparable conditions are as yet available for gains made on daily doses of pure carotene lower than 0.001 mg., extrapolations for the smaller fruit doses are not attempted.

Careful determination of a sufficient number of points upon the pure carotene-weight gain curve may yield a formula from which the probable vitamin A activity of foods of known carotene content may be estimated. Data bearing on this possibility are now being collected. It is of course recognized that variations in absorbability of the carotene may occur which would render the use of such a method only secondary to the biological test. A corol-

lary to this observation may be found in the vitamin A and carotene stores found in the livers of rats fed the various doses. An attempt is now under way in this laboratory to determine such stores as well as the carotene of the feces in the hope of establishing carotene and vitamin A balances.

*Failure of Correlation between Total Carotenoid and Provitamin A Content of Tomatoes*—As shown in Fig. 3, of the outdoor normally vine-ripened tomatoes the deep red variety, Gigante Ingretnole, appeared to provide the largest amount of vitamin A activity, fol-

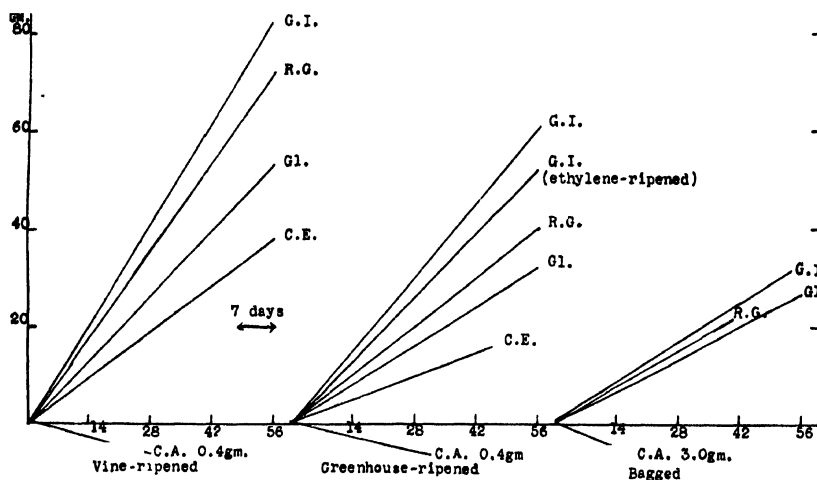


FIG. 3. Rate of growth of vitamin A-free rats fed 0.1 gm. daily doses of five varieties of tomatoes ripened under different conditions. Larger doses of the pale yellow tomato, Clark's Albino, were fed. G. I. indicates Gigante Ingretnole; R. G., Ruby Gold; Gl., Globe; C. E., California Earliana; C. A., Clark's Albino.

lowed by deep yellow Ruby Gold, the red Globe, and medium red California Earliana in the order named. The pale yellow Clark's Albino was practically free from precursors of vitamin A. The same order was noted for the vitamin content of the fruits ripened under glass in the greenhouse and also for the bagged fruits. In all cases the greenhouse vine-ripened fruits were lower in vitamin activity than the corresponding outdoor products and the bagged fruits lowest of all. Smaller differences were seen moreover among the values shown by bagged specimens of the three varieties,

Gigante Ingretnole, Ruby Gold, and Globe, than by the corresponding outdoor and greenhouse normally ripened fruits.

The same procedure was used in determining the carotenoid content of the tomatoes as of the tree fruits. However, since lycopene could not be separated from carotene by any of the extraction methods available when this work was done, a correlation of vitamin A activity with carotenoid content thus obtained is not possible. The tomato pigment figures are not quoted here therefore, although some obvious differences may be mentioned. Kuhn and Brockmann (8) have recently reported a method of separation of the carotenoids in which lycopene and carotene in benzene solution are separated by means of adsorption of the former on fibrous aluminum oxide. This method should be readily adaptable to the study of the pigments of tomatoes. In any case, since von Euler, von Euler, and Karrer (4) and also Steenbock and Schrader (16) have reported purified isolated lycopene to be ineffective as a precursor of vitamin A, no clear correlation between carotenoid and provitamin A values of tomatoes can be expected to emerge from any estimations in which the separation was not effected.

The Ruby Gold tomatoes grown in the greenhouse and bagged were a deep reddish orange as contrasted with the deep yellow fruit ripened outdoors on the vines. The vitamin A activity was somewhat reduced although the pigment content was apparently increased. If the entire carotenoid pigment obtained in the outdoor vine-ripened fruits, 0.03 mg. per gm. of fruit, be considered as carotene, its growth-promoting value may be seen to fall into the same area as did those of the yellow tree fruits. The 50 mg. daily doses of Ruby Gold tomato, providing 0.0015 mg. of carotene daily, allowed a gain of 68 gm. in 56 days, or 0.81 gm. per 0.001 mg. of carotene and the 100 mg. doses 72 gm., or 0.43 gm. per 0.001 mg. This is in excellent agreement with the other values shown in Fig. 2. The increased pigmentation, up to 10 times that of the normally vine-ripened, seen in our greenhouse-ripened and bagged samples of Ruby Gold was probably due to the production of lycopene which may have been accompanied by a decrease in carotene formation. Certainly there is no correlation between their total carotenoid content and growth of vitamin A-free rats similar to that seen in the yellow tree fruits.

In the green sample only could carotenoid pigment or vitamin

A activity be detected in the Clark's Albino, a very pale yellow tomato. The Globe tomato showed no change in carotenoid content due to ripening under glass although its vitamin activity was reduced as in the other varieties. A measurable decrease was observable in the carotenoid and vitamin value of the bagged fruit. Similar decrease in both characteristics is seen in the California Earliana tomato ripened under glass. The deep red Gigante Ingretnole, however, had an obvious increase in depth of color of fruits ripened under glass and in ethylene although no corresponding rise in vitamin A was seen. Again no correlation between total pigmentation and biological activity is discernible.

*Chlorophyll and Provitamin A*—It is clear that in the total absence of light both carotene and lycopene are developed in the red and yellow tomatoes and carotene in the yellow tree fruits. Chlorophyll was never present in the bagged tomatoes, the color proceeding from white through pink to red. Chlorophyll is seen, therefore, not to be a necessary predecessor of the carotenoids, at least *in situ*, nor has the presence of chlorophyll any predictable relation to vitamin A activity. This indicates the erroneous character of the supposition of Lubimenko (9) and of Tobler and Tobler (17) that the lycopene and lycopeneoids of tomatoes are produced by the decomposition of chlorophyll. It is likewise not in line with the conclusions of Heller (5) nor with the theory of Schertz (13) as to the interrelation of chlorophyll and vitamin A precursors. The work of Crist and Dye (3) in support of the association of vitamin A precursors with greenness in plants would appear to be better interpreted as indicating the frequent but not invariable association of carotene with chlorophyll. Indeed the conditions laid down by these authors for a demonstration of the lack of relationship between chlorophyll and vitamin A have been realized in the experiments here reported. They state that, "The present authors, who think the reverse of this is true [that is, Bezssonoff's (2) theory that vitamin A is a conditioner of chlorophyll formation], are unwilling to concede the point prior to a time when plant tissue which is entirely devoid of chlorophyll and yet abundant in vitamin A shall have been realized and its vitaminic potency demonstrated in terms of adequate gains in weight by animals fed thereon." Our previously published report (12) of the low content of vitamin A in green tomatoes and in green

peppers as compared with the ripe red specimens is further evidence of the irrelevance of the occurrence of chlorophyll in this respect.

The question of the necessity for the presence of chlorophyll in other parts of the plant if not in the ripening fruit in order to insure the production of carotene or other vitamin A precursors appears adequately answered by the normal development of vitamin activity in plucked fruit. Tomatoes develop the red or yellow color and the ability to promote the growth of vitamin A-depleted rats when ripened off the vine, in the dark, in diffused light, or in ethylene. Similar vitamin development occurs in bagged tomatoes ripened on the vine without benefit of light or chlorophyll. It would seem necessary therefore to seek elsewhere than in chlorophyll for the precursors of both carotene and lycopene.

*Effect of Ethylene Ripening*—Some interest attaches to the data on ethylene-ripened tomatoes which are shown in Fig. 3. Samples of Gigante Ingretnole ripened in ethylene gas (1:2000) developed an extremely deep red color and carotenoid value larger than was shown by any of the other tomato fruits examined. The vitamin activity was slightly less, however, than that of the less deeply colored vine-ripened fruit. It is possible that the proportion of lycopene to carotene was increased in the ethylene-ripened fruit. The ethylene-ripened tomato previously reported (12) as showing normal vitamin A value was the San Jose Canner, a red fruit quite similar in color to the California Earliana with which it was nearly identical in growth-promoting property. House, Nelson, and Haber (6) using a much larger dosage of the Bonny Best variety confirmed our finding on the development of vitamin A activity in plucked tomatoes. Jones and Nelson (7) found lowered vitamin A in ethylene-ripened tomatoes but used only the strained juice, which has been shown by Steenbock and Schrader (16) to contain only a small part of the vitamin A of tomatoes.

Further proof of the relatively regular rate of increase in vitamin A content during the ripening process was shown by feeding fruits of the Globe variety picked at the mature green stage, the half-ripe stage, when the fruit had lost most of its green color, the three-quarters ripe stage, when the fruit was not quite uniformly colored, and the ripe or completely colored fruit. These results are shown in Fig. 4. The pigment of the green fruit was found to contain chlorophyll and xanthophyll as well as carotene while the ripening



portions undoubtedly contained lycopene and carotene. Thus the undifferentiated pigment values in tomatoes may be only roughly proportional to the growth-promoting property and the increase in vitamin A value is seen to occur parallel with the disappearance of the green color when the fruit ripens on as well as off the vine.

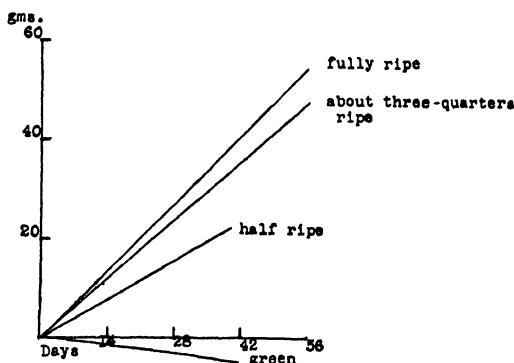


FIG. 4. Rate of growth of vitamin A-free rats fed 0.1 gm. daily doses of Globe tomato plucked at various stages of ripeness.

#### SUMMARY

1. Royal apricots, white and yellow peaches, white and yellow nectarines, and five varieties of tomatoes ripened under various light conditions were studied as to content of total petroleum ether-soluble pigments and vitamin A activity.

Xanthophyll was found only in the green fruits and carotene only was present in the apricots, yellow peaches, yellow nectarines, and possibly the deep yellow variety of tomato. The three red varieties of tomato undoubtedly contained lycopene as well as carotene but no separation of these pigments was effected. The white peaches, white nectarines, and pale yellow tomato contained no measurable amounts of carotenoids and were likewise nearly devoid of vitamin A value.

2. The vitamin A activity and carotene content of the yellow peach was slightly increased in fruit bagged in the blossom stage over that seen in the normally tree-ripened fruits. A slight decrease in both characteristics, however, was seen in the bagged

apricots and yellow nectarines. Chlorophyll was not present at any time in the bagged fruits.

3. All of the greenhouse-grown tomatoes were slightly inferior in vitamin value to those grown outdoors, and bagged tomatoes were somewhat inferior to both but by no means devoid of vitamin A in spite of the absence of chlorophyll throughout the whole period of development. The decrease in vitamin value due to absence of light was more noticeable in the tomatoes than in the apricots and yellow nectarines.

4. The carotenoid content of the greenhouse and bagged tomatoes was in some cases obviously greater than that of the outdoor vine-ripened but with the exception of the deep yellow variety; judged by vitamin activity this must be assumed to be largely due to an increase in a biologically inactive pigment, probably lycopene.

5. A relationship which approximates a logarithmic curve appears to exist between the total growth of vitamin A-free rats per unit of carotene ingested in the 56 day experimental period and the level of daily carotene intake. The values found for crystallized carotene, apricots, yellow peaches, and yellow nectarines fall close to one another on such a curve.

#### CONCLUSIONS

1. Chlorophyll is not a necessary intermediary *in situ* for the formation of carotene, lycopene, or any other possible precursors of vitamin A.

2. Fruits which develop carotene and vitamin A activity under normal light conditions do so also under glass and in the dark although usually in slightly smaller amounts.

3. Lycopene is probably not a precursor of vitamin A.

4. A constant relation exists between level of daily intake of carotene by vitamin A-free rats and their growth per unit of total carotene ingested. This relation illustrates the "law of diminishing returns."

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## GINGIVAL TISSUE LIPIDS

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"The gum [gingiva] is a smooth, firm, pale pink tissue around the necks of the teeth, continuous externally with the sulci between the lips and cheeks, and internally with the hard and soft palates, floor of the mouth and root and sides of the tongue. The minute anatomy may be conveniently considered under the following heads: (A) the mucous membrane; (B) the sub-mucous tissue. The mucous membrane, about 0.3 mm. thick, [in health] is essentially of a stratified epithelial character, consisting . . . of several layers. . . The sub-mucous tissue consists of dense bundles of connective tissue. . . Other constituents of the subepithelial tissue are:— (i) small lobules of fat cells . . . (ii) mucous glands, . . . (iii) blood vessels, . . . (iv) . . . nerve fasciculi; and (v) the so-called 'glands' of Serres" (1).

As far as is known no analyses have been heretofore reported on the lipid constituents of gingival (gum) tissue. Therefore, it was purposed to analyze gingival tissues from healthy, diseased, and edentulous mouths to find the lipid contents, to observe the relation to other more or less similar tissues, and to determine whether common oral diseases gave a significant lipid content change.

In a recent review of skin lipids, Kooyman (2) points out the meager literature existing. Values for skin phospholipids have been reported, however, by Eckstein and Wile (3) as approximately 0.2 per cent (moist weight). Total lipids in the skin are given (a) by Klose (4) as the ether-soluble fraction averaging 25.0 per cent but varying from 0.1 to 62.0 per cent (moist weight) depending on the constitution of the adhering subcutaneous tissue; (b) by Eckstein and Wile (3) as the ether-soluble fraction varying from 2.4 to 9.9 per cent (moist weight); and (c) by Jono (5), averaging 2.22 per cent and varying from 1.95 to 2.51 per cent by the Kawaguchi-Suto method. Skin cholesterol analyses have been

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reported by Unna and Golodetz (6), Rosenbaum (7), Kawaguchi (8), Eckstein and Wile (3), Valentin (9), Bürger and Schlomka (10), Roffo (11), and Jono (5). These investigators give values for human skin ranging from 0.1 to 0.4 per cent (moist weight); of guinea pig skin from 0.4 to 0.6 per cent (moist weight). The percentage residue (dry extracted weight) of skin has been reported by Klose (4), Kawaguchi (8), and Bürger and Schlomka (10), as varying from 20.46 per cent in infant tissue to 39.8 per cent (moist weight) in adult tissue.

Intestinal mucosa lipids have been studied by several investigators. Mucosa phospholipids have been reported by Sinclair (12) as 10.41 per cent of the dry weight of the tissue, and (unpublished results) he has also found an average of 1.92 per cent (moist weight) of acetone-insoluble fraction in normal cat mucosa. Total lipids of the intestinal mucosa have been reported by Ewald (13), Moore (14), and Noll (15), who give values ranging from 3.6 to 5.8 per cent (dry weight). Sinclair (unpublished data) has found 0.66 per cent (moist weight) or 4.04 per cent (dry weight) of acetone-soluble fraction. Cholesterol of the intestinal mucosa has been reported by Mueller (16) as being of the order of 0.2 per cent (moist weight). The residue obtained upon extracting and drying the mucosa has been reported by Noll (15) as 13.56 to 16.50 per cent (moist weight), whereas Sinclair (unpublished data) found an average of 16.4 per cent (moist weight) from twelve control cats in the postabsorptive state.

About 95 samples of upper or lower gingival tissues were taken at autopsy. The tissue samples removed extended usually from bicuspid to bicuspid in the maxilla and from the first molar to the lateral incisor on each side of the mandible.

The extraction was carried out by a suitable adaptation of the method described by Bloor (17). To check completeness of extraction, saponification of the residue after extraction on four samples gave lipid material recovered as 0.26, 0.20, 0.13, and 0.25 per cent (moist weight) by an oxidative method. The methods used for the analyses were standard microprocedures for lipids (18); cholesterol was determined colorimetrically, and phospholipid and fatty acids by oxidation.

The data obtained are presented in Table I, in Column 1 of which is given the sample number, upper or lower gingiva being

TABLE I  
*Lipid Content of Gingival Tissue*

Sample No*	Age	Moist weight, approximate	Cholesterol	Phospholipid	Total lipid	Cholesterol after saponification	Residue†
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	yr	gm	per cent	per cent	per cent	per cent	per cent
35 U	3½	0 11		1 44	2 25		23 7
35 L.	3½	0 15		1 43	1 88		22 8
36 U.	5 mo.†	0 32		0 54	1 49		12 3
37 "	6 " †	0 34		0 82	1 84		12 2
38 "	49	1 13	0 21	0 20	2 25		22 9
38 L	49	0 48	0 26	0 79	3 25		21 1
39 U.	44	0 23		1 73	2 45		23 0
40 "	Term	0 75	Nil§	0 30	0 76	0 10	18 2
41 "	7 mo.†	0 25	" §	1 06	1 64	0 11	17 5
41 L.	7 " †	0 29	0 03§	0 61	3 20	0 16	16 0
42 U.	12 days	0 15	0 09§	0 98	1 11	0 17	17 6
42 L	12 "	0 20	Nil§	1 16	1 35	0 12	19 0
43 U.	6 mo †	0 49	0 03§	0 46	0 74	0 14	11 5
43 L.	6 " †	0 34	0 05§	1 03	1 30	0 16	14 4
44 U.	6 " †	0 38	Nil§	0 83	1 39	0 16	14 0
44 L	6 " †	0 37	" §	1 06	1 61	0 16	12 5
45 U	20	0 60	0 20	1 26	1 35		23 5
45 L	20	0 11		1 58	2 62		19 6
46 U	82	1 18	0 15	1 41	1 46	0 24	22 5
47 "	56	0 46	0 26	1 31	2 83		23 5
47 L	56	0 13	0 49	1 86	3 56		23 8
48 U	46	0 46	0 18	1 17	1 68	0 45	19 4
48 L	46	0 10		1 26	2 16		23 3
49 U	52	0 45	0 22	1 24	2 98		22 8
49 L	52	0 21	0 38	1 73	2 33		21 2
50 U	49	0 37	0 27	1 22	1 69		25 9
50 L.	49	0 09		2 00	2 62		22 0
51 U	6 mo.†	0 14		0 90	2 11		12 7
51 L	6 " †	0 19		0 39	0 79		12 8
52 U	8 " †	0 29		0 71	1 56		15 2
52 L	8 " †	0 22		0 80	0 89		14 2
53 U	7 " †	0 50	0 25	0 44	0 33		18 8
53 L	7 " †	0 31		0 60	1 19		17 2
54 U	55	0 54	0 27	0 58	1 21		18 8
54 L	55	0 23		0 79	0 79		25 4
55 U.	75	0 58	0 25	0 66	0 90		25 8
55 L.	75	0 06		1 47	2 84		25 7

TABLE I—*Concluded*

Tissue		Cholesterol	Phospholipid	Total lipid	Cholesterol after saponification	Residue†
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Total	No. of analyses	40 00	97	96	26	37
	Average	0 20	0 95	2 15	0 16	19 3
	Standard deviation	0 08	0 74	1 65	0 07	4 42
Infant	No. of analyses	17	50	46	17	17
	Average	0 14	0 64	2 12	0 13	15 1
	Standard deviation	0 05	0 27	1 88	0 03	2 47
Adult	No. of analyses	23	45	44	9	20
	Average	0 24	1 19	2 34	0 21	22 8
	Standard deviation	0 02	0 94	1 30	0 09	2 01
Upper	No. of analyses	26	54	55	16	21
	Average	0 19	0 88	1 99	0 17	19 1
	Standard deviation	0 06	0 78	1 61	0 08	4 61
Lower	No. of analyses	14	41	40	5	16
	Average	0 21	1 01	2 44	0 14	19 4
	Standard deviation	0 12	0 70	1 40	0 05	4 29
Infant upper	No. of analyses	10	28	28	9	10
	Average	0 15	0 61	1 97	0 14	15 0
	Standard deviation	0 05	0 27	1 56	0 03	2 67
Infant lower	No. of analyses	7	22	21	8	7
	Average	0 13	0 68	2 34	0 12	15 2
	Standard deviation	0 05	0 28	1 54	0 03	2 20
Adult upper	No. of analyses	16	25	25	7	11
	Average	0 22	1 28	2 11	0 22	22 9
	Standard deviation	0 05	1 02	1 45	0 10	2 06
Adult lower	No. of analyses	7	19	18	2	9
	Average	0 29	1 39	2 67	0 17	22 8
	Standard deviation	0 10	0 89	1 20	0 03	1 89

\* Data on the first thirty-four autopsies are omitted for brevity

† By residue is meant the dried extracted tissue residue

‡ Premature.

§ Free cholesterol.

|| Denotes approximate only colorimetric measurements

denoted by U. and L. respectively; in Column 2, the approximate age of the patient; in Column 3, the moist weight<sup>1</sup> of the tissue sample; in Columns 4 to 7, the percentage content (moist weight) of cholesterol, phospholipid, total lipid, and cholesterol after saponification; in Column 8, the per cent of dry extracted residue

<sup>1</sup> The moist weights were determined precisely to the mg

(moist weight). The determinations of cholesterol after saponification were made on aliquots which had been saponified to ascertain possible effects of that process on cholesterol.

A few determinations were made of free cholesterol (Column 4). The cholesterol was determined on a suitable aliquot. The free cholesterol was precipitated by the digitonide method from a fresh aliquot and cholesterol determined colorimetrically in the filtrate. The difference was called free cholesterol.

Although the variations in the percentages (moist weight) of total lipid, phospholipid, and cholesterol may be described as irregular, it is noted that in most cases the lipid content of the lower gingivæ is greater than that of the upper.

In the lower part of Table I are presented the data calculated from the experimental results showing for each class of lipids the number of analyses, the average, and the standard deviation<sup>2</sup> for all analyses, for infant, adult, upper, lower, infant upper, infant lower, adult upper, and adult lower tissues. It may be noted that the average value for phospholipid in infants is nearly identical for upper and lower gingivæ, while for adults the values for both upper and lower are substantially higher than for infants. The average phospholipid content for adult lower gingivæ is somewhat larger than for upper gingivæ although the magnitude of this difference is insignificant mathematically but probably real. For total lipids and also for cholesterol the average values are greater in lower gingivæ than in upper gingivæ.

The difference between lipid content of upper and from lower gingivæ from the same mouth was calculated and gave on the average +0.60 per cent for total lipids, +0.65 per cent for phospholipids, and +0.10 per cent for cholesterol. These are all seen to be positive, but calculation of standard deviations of differences permits little importance to be attached to the magnitude of the positive difference.

The average values for cholesterol before saponification were: upper gingiva, 0.23 per cent; lower, 0.30 per cent; and after saponification: upper, 0.18 per cent; lower, 0.27 per cent. In spite of the apparent change in the lower tissue samples the calculation of standard deviations showed the difference to be

<sup>2</sup> Calculations of the average deviation from the average gave no additional information and are omitted.



negligible. The average difference for all tissue samples is  $+0.002$  mg. of cholesterol (before saponification minus after).

There are sixteen pairs of cholesterol determinations in which the values before and after saponification are considered exact. These give average values of 0.13 per cent before and 0.19 per cent after, a difference possibly attributable to the uniformly smaller aliquots for the determinations after saponification. A careful study of the cholesterol determinations showed a close inverse correlation between the size of aliquot and the figure obtained for per cent of cholesterol.

The percentage extracted weight shows an average value of 15.1 per cent residue for infant tissue and 22.8 per cent residue for adult tissue. The standard deviation from these average values is 2.47 for infant and 2.01 for adult tissue, indicating that the percentage residue is substantially different in infant than in adult gingivæ.

In comparing the lipid content of skin, intestinal mucosa, and gingival tissue, it may be seen that the phospholipid content of the gum is much more nearly allied to the mucosa than to the skin, lying, however, between these latter two in value. The values for total lipids show the average value in the gum to be of the order of Jono's (5) reported value for skin and sensibly greater than the values reported for mucosa, lying between the skin and mucosa in per cent of content. The cholesterol content of the gum lies in the limits reported for skin cholesterol but is only slightly larger than the values quoted for mucosa. It is interesting to note that the residue percentage for infant gingival tissue is closely comparable to the residue from mucosa and lower than that of infant skin, while the adult gingival tissue gave values close to those of skin and much higher than those for mucosa. The lipid content of gum lies between that of skin and that of intestinal mucosa, the phospholipids being nearer to those of mucosa, and the total lipids and cholesterol are more nearly like those values reported for skin.

In conjunction with these analyses an anatomical and histological study was made by Dr. W. McL. Davis,<sup>\*</sup> which embraced the cause of death, gross diagnosis of mouth conditions, and a comparison of tissues prepared for microscopic examination. No

<sup>\*</sup> Rockefeller Fellow in Dentistry at this school.

correlation was found between cause of death, histological structure, or health of the mouth and the lipid content.

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## THE DEPOSITION AND UTILIZATION OF HYDROGENATION ISOOLEIC ACID IN THE ANIMAL BODY

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It has been demonstrated by many observers that the composition of the body fat of animals is largely dependent upon the composition of the diet, and particularly upon that of the dietary fat. Lebedev (1), after fasting dogs until their reserve fat was exhausted, found that feeding linseed oil caused the deposition of a reserve fat having a melting point below 0°; while feeding mutton fat produced a reserve fat melting at about 50° or much higher than normal dog fat. Henriques and Hansen (2) fed hogs on barley and corn, respectively, and found the melting point of the lard to be lower and the iodine value higher in the corn-fed animal, as would be expected from the greater unsaturation of corn oil. A demonstration of this principle on a very large scale has been given recently in the course of investigations dealing with the "soft pork" problem (3). The greater the amount of food containing unsaturated fats (for example peanuts) which is fed to the hogs, the greater the tendency to the production of "soft pork" and oily lard.

It has also been shown that it is possible for fatty acids which are not normally present in the body to be deposited in the adipose tissue when fats containing such acids occur in the food. Winternitz (4) obtained storage of iodized fatty acids by feeding iodized fat, and Munk (5) isolated erucic acid from the fat of a dog which had been fed rape-seed oil. There is, however, no evidence to show whether or not such abnormal fatty acids can be utilized by the body in the course of its metabolism.

At the present time, very large amounts of vegetable and marine animal oils are partially hardened by catalytic hydrogenation and

consumed as human food, chiefly in the form of vegetable shortenings (lard substitutes) and margarine, and baked goods of which the above are constituents. Such shortenings may contain 30 per cent or more of an isomer (or mixture of isomers) of oleic acid which is formed during the course of the hydrogenation process. This substance, which is usually termed "isooleic acid," is solid at ordinary temperatures, melts at about 44°, and yields a lead salt which is relatively insoluble in ether, alcohol, and the other common organic solvents. It thus resembles the saturated acids in its properties. The exact composition of isooleic acid is still uncertain, but it would seem to consist largely of elaidic acid (*trans*  $\Delta_{9:10}$  octadecenoic acid), also probably some  $\Delta_{12:13}$  acid resulting from partial hydrogenation of linoleic acid, and possibly still other isomers of oleic acid. A discussion of the investigations dealing with its composition has been given by Ellis (6).

Since isooleic acid is not known to occur as a constituent of any natural fat, and since such large quantities are used as human food, it seemed of considerable interest to determine its behavior in the animal body, and its probable effects in human nutrition. It is interesting to note, however, that in their earliest attempts to justify the use of hydrogenated fats as food, several observers studied the digestion of hydrogenated fats, and found their digestibility equal to that of natural fats (7-11).

A consideration of the possible treatment of dietary isooleic acid by the body indicates four possibilities. (a) The acid may be rejected by the body and excreted. In this case it should not appear in the body fat, and should be concentrated in the feces. (b) It may be absorbed and deposited in the body fat as such. In this case it should be recoverable from the body fat, with no concentration taking place in the feces. (c) It may be absorbed and converted into liquid oleic acid, or some other compound, before being deposited or utilized. In this case it should neither be concentrated in the feces nor appear in the body fat. (d) It may be absorbed and deposited, but not utilized. If it is deposited, the simplest test of its utilization would seem to be the effect of subsequent fasting. If the acid is metabolized, it should disappear; if its utilization is impossible, it should either become concentrated in the body fat during fasting, or be excreted and appear in the feces.

To test the above possibilities, the experiments to be described were devised.

#### EXPERIMENTAL

*Deposition of Isooleic Acid in Body Fat*—The albino rat was selected as the most suitable animal for the investigation, since it will tolerate high concentrations of fat in its diet. Four adult rats were fasted until their weights had been reduced to about 80 per cent of the original level. The animals were then placed on the following diet, which is a modification of that used by Powell (12).

	per cent
Casein (commercial)	10
Sucrose . .	5
Osborne and Mendel salt mixture*	4
Sodium chloride. . .	1
Corn-starch. .	40
Hydrogenated cottonseed oil	40
	—
	100

\* Osborne, T. B., and Mendel, L. B., *J Biol. Chem.*, **37**, 572 (1919).

The diet was fed *ad libitum*, with tap water *ad libitum* and about 5 gm. of fresh spinach daily per animal, and feeding was continued until the original weight had been approximately regained. The animals were then killed and analyzed. As controls, a second group of five rats were treated in exactly the same way as the test animals, but received lard in place of the hydrogenated oil.

The hydrogenated oil used was a sample prepared in the laboratory from refined cottonseed oil. The oil was hydrogenated with a nickel catalyst to approximately the consistency and iodine value of lard, under conditions designed to yield a product with a high content of isooleic acid. The analyses of the product and of the lard used as control are given in Table I. The "slipping point" which is a measure of the melting point of the fat, was determined by a modification of the method of Whitner and Bailey (13).

The presence or absence of isooleic acid in the body fat was determined as follows: The total fatty acids in the body were obtained by saponification and extraction by a modification of the method of Liebermann as described by Leathes and Raper (14).

The procedure was as follows: The rats were electrocuted and the carcasses placed in 1000 cc. Pyrex beakers. An aqueous solution of caustic potash containing 40 gm. of KOH per 100 cc. of solution was added to each beaker in the proportion of 1 cc. of solution per gm. of carcass weight. The beaker was heated in a boiling water bath for one-half hour. A volume of 95 per cent ethyl alcohol equal to the volume of caustic solution used was then added, and the heating continued for 1 hour longer to complete the saponification of the fat. The product was washed into a 2000 cc. volumetric flask with hot water, and the volume completed; 25 cc. samples

TABLE I  
*Composition of Fats Used in Test Diets*

	Hydrogenated oil	Lard
Iodine value	63.3	63.7
Refractive index, 40° (Zeiss butyro-refractometer)	51.0	50.5
Slipping point (Bailey-Whitner), °C.	37.0	42.1
Composition of fatty acids (Baughman-Jamieson method)		
Iodine value of mixed fatty acids	66.3	66.7
Solid acid fraction, per cent	53.7	36.1
Iodine value, solid acid fraction	43.5	1.0
" " liquid " " (calculated)	92.6	103.9
Calculated		
Saturated acids, per cent	27.7	35.7
Isooleic acid (apparent), per cent	26.0	0.4
Oleic acid, per cent	45.0	54.2
Linoleic acid, per cent	1.3	9.7

were removed with a pipette for the quantitative estimation of fat, in which the procedure of Leathes and Raper was followed exactly.

The remainder of the solution in the volumetric flask was washed out into a 6 liter Florence flask, and acidified by the addition of sulfuric acid solution containing 40 per cent  $H_2SO_4$  by volume, in the proportion of 1.5 cc. per gm. of carcass weight. The mixture was cooled, 600 cc. of petroleum ether added, and the whole shaken thoroughly and allowed to stand overnight.

The petroleum ether solution was decanted as completely as possible and filtered. About 5 gm. of activated charcoal were

added to remove coloring matter, and the solution was again filtered, yielding a water-white filtrate. The petroleum ether was removed by distillation in a current of  $\text{CO}_2$ , and the sample dried in an oven at  $105^\circ$  in a current of  $\text{CO}_2$ . The fatty acids recovered were pale yellow in color. A sample of the mixed fatty acids obtained was separated into "solid" and "liquid" fractions by crystallizing the lead salts from alcohol by the method of Twitchell (15) as modified by Baughman and Jamieson (16). The solid fraction consists of the saturated acids and the isooleic acid, since

TABLE II  
*Feeding Records*

Fasting time, 5 days; feeding time, 18 days

	Rat No and sex	Initial weight	Weight after fasting	Weight after feeding	Fat content
		gm	gm	gm	per cent
Controls, lard diet	11 ♂	200	151	201	12.4
	12 ♂	276	209	289	20.3
	14 ♀	174	133	189	14.1
	15 ♀	194	149	198	13.9
	17 ♀	173	129	183	14.1
Average					15.0
Hydrogenated oil diet	19 ♀	214	163	199	13.0
	20 ♀	140	103	143	13.0
	21 ♀	182	137	163	10.0
	23 ♀	208	167	208	13.6
Average					12.4

these acids yield insoluble lead salts. The iodine value of the solid acids was then determined by the method of Wijs (17). Theoretically, this iodine value should be due entirely to isooleic acid, since the saturated acids have no iodine value. Actually, however, it is found that all natural fats yield a solid acid fraction which has a small iodine value (usually less than 3.0) due to contamination of the precipitated lead salts with small amounts of lead oleate, which persists after recrystallization. However, any significant increase over the controls in the iodine value of the solid acids from the animals fed hydrogenated oil conclusively



indicates deposition of isooleic acid in the fat of the latter group. The iodine value of isooleic acid was taken as 90.0.

The results of the feeding tests are given in Table II. The analyses of the body fats of the animals are given in Table III.

The feces of the animals fed on the hydrogenated oil diet were collected over the whole feeding period, combined, and a sample analyzed by the same procedure used for the body fat. The results are shown in Table IV, first column. Comparison of this

TABLE III  
*Analysis of Mixed Fatty Acids from Body Fat*

	Rat No	Iodine value, mixed fatty acids	Solid acid fraction	Iodine value, solid acid fraction	Apparent isooleic acid
			<i>per cent</i>		<i>per cent</i>
Controls, lard diet	11	75 6	29 3	2 4	0 8
	12	75 2	28 6	2 2	0 7
	14	76 3	29 3	3 4	1 1
	15	73 3	27 0	4 0	1 2
	17	68 9	26 6	2 5	0 7
Average . . . . .		73 9	28 2	2 9	0 9
Hydrogenated oil diet	19	75 2	32 1	20 6	7 4
	20	75 2	34 2	23 3	8 8
	21	70 0	35 4	27 0	11 6
	23	75 6	33 8	24 4	9 2
Average . . . . .		74 0	33 8	23 8	9 2

Increase of apparent isooleic acid over controls = 8.3 per cent of total fatty acids.

Average isooleic acid as percentage of body weight = 1.04 per cent.

analysis with the composition of the dietary fat shows that there has been no concentration of isooleic acid in the feces, but rather the reverse.

It is evident from these results that isooleic acid may be absorbed into the body, and that some, at least, of the absorbed acid may be deposited unchanged in the body fat. (The possibility of the transformation of some of the acid into liquid oleic acid during absorption is not excluded.) The iodine values of the solid acid fractions from the test animals are almost 10 times

greater than the control values, and indicate that about 8 per cent of the body fat of the former group consists of isooleic acid. No concentration of the acid takes place in the feces, and it is concluded that there is no greater tendency for the body to reject this than any other fatty acid.

*Utilization of Deposited Isooleic Acid*—Twenty-four adult albino rats were divided into five groups, and fasted until their weight had been reduced to about 80 per cent of the original level. Three groups were then fed the diet containing hydrogenated oil, and two groups the diet containing lard, until the original weight was approximately regained. The rats in one group receiving hydro-

TABLE IV  
*Analysis of Feces from Animals Fed Hydrogenated Oil Diets*

	First test	Second test (second fast period)
Total weight of feces, gm	52 0	25 5
Fat content, as fatty acids, per cent	20 0	16 1
Composition of fat		
Iodine value, mixed fatty acids	67 7	58 5
Solid acid fraction, per cent	49 7	43 9
Iodine value, solid acid fraction	30 6	21 7
Apparent isooleic acid, per cent	16 9	10 6
Total weight of isooleic acid excreted by all animals, gm		0 435
Approximate weight of isooleic acid which dis- appeared from body fat during fast period, gm . . . . .		19 2

genated oil were then killed and analyzed, as controls on the isooleic acid content of the other two groups receiving this diet. The remaining four groups were then again fasted to 80 per cent of the initial weight, and their feces collected during this period to determine whether or not excretion of isooleic acid had taken place. At the end of this second fasting period the rats in one group on each diet were killed and analyzed to determine whether the isooleic acid content of the body had been depleted in proportion to the total fat content. The two remaining groups were then again fed until the original weight was again approximately regained. The purpose of this second feeding period was in case

the isooleic acid content was not removed by fasting, to show whether the concentration in the body could be further increased by further feeding.

The average feeding records of the five groups are shown in Table V. The analyses of the body fat samples are given in Table VI. In order to economize space, the average results and standard deviations only are given. The standard deviation,

TABLE V  
*Feeding Records*

Group No.	Diet	No of animals	Average weight					Average fat content
			Initial	After first fast	After first feeding	After second fast	After second feeding	
			gm	gm	gm	gm	gm	per cent
1	Hydrogenated oil	5	205	150	179	Killed after first feeding		11.0
2	Lard	5	230	175	213	176	Killed after second fast	2.9
3	Hydrogenated oil	5	215	158	203	168	" "	2.2
4	Lard	5	189	138	172	139	186	13.9
5	Hydrogenated oil	4	203	148	176	141	186	11.9

tion,  $\sigma$ , is calculated as recommended by the American Society for Testing Materials (18) from the formula,

where  $d$  = deviation of an individual result from the average and  $n$  = number of results.

The feces of the animals fed hydrogenated fat were collected during the second fasting period and analyzed. The results are shown in Table IV, second column.

The results indicate conclusively that isooleic acid is utilized in the body; in fact, it is utilized somewhat more readily than the

average of the body fatty acids, since the concentration in the body fat after fasting (Table VI) was only 7.7 per cent, as compared to about 11 per cent before fasting (compare Groups 1 and 3). The acid is not excreted in the feces since the total fecal isooleic acid from the nine animals in Groups 3 and 5 is only 0.435 gm., whereas, approximately 19.2 gm. have disappeared from these animals. (This last figure was arrived at by assuming 11 per cent of fat containing 11.4 per cent of isooleic acid in these animals before fasting, as indicated by the results of Group 1)

TABLE VI

*Average Results and Standard Deviations Obtained on Analysis of Mixed Fatty Acids from Body Fat*

Group No	No of animals	Diet	Killed after	Iodine value, total fatty acids	Solid acid fraction	Iodine value, solid acid fraction	Apparent isooleic acid	Excess isooleic acid over controls
					per cent		per cent	per cent
1	5	Hydrogenated oil	First feeding	78.1 ( $\sigma$ 1.3)	38.2 (1.0)	26.9 (1.4)	11.4 (0.7)	About 10.6
2	5	Lard	Second fast	80.9 ( $\sigma$ 2.1)	25.9 (0.8)	3.1 (0.4)	0.9 (0.1)	
3	5	Hydrogenated oil	" "	82.4 ( $\sigma$ 4.3)	31.8 (1.2)	21.8 (1.8)	7.7 (0.8)	6.8
4	5	Lard	Second feeding	76.6 ( $\sigma$ 2.8)	28.8 (0.6)	2.1 (0.1)	0.7 (0.1)	
5	4	Hydrogenated oil	" "	74.7 ( $\sigma$ 1.6)	36.1 (0.7)	25.5 (0.5)	10.2 (0.2)	9.5

Also, there is no tendency for isooleic acid to accumulate in the body on repeated fasting and feeding, since the concentration of the acid in the rats of Group 5 is slightly less than that in Group 1. It may be concluded that isooleic acid may take part in the fat metabolism of the body without any greater difficulty than any other dietary fatty acid, and that it is in no way objectionable as a constituent of foodstuffs.

## SUMMARY

1. When fasted animals are fed a diet containing partially hydrogenated cottonseed oil, isooleic acid derived from the oil appears as a constituent of their body fat.

2. This deposited isooleic acid disappears during subsequent fasting at least as readily as the other fatty acid constituents of the body fat.

3. It is concluded that isooleic acid may be utilized as fuel by the animal body to the same extent as other dietary fatty acids, and that it is in no way objectionable as a dietary constituent.

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# THE EFFECT OF THE INGESTION OF SODIUM, POTASSIUM, AND AMMONIUM CHLORIDES AND SODIUM BICARBONATE ON THE METABOLISM OF INORGANIC SALTS AND WATER\*

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The factors which govern the state of hydration of the tissues and the metabolism of water have been the subject of a number of recent investigations. Numerous experiments have been devised to determine the cause of water retention in edema, and a few have been directed at the variation in water content of normal individuals. Numerous theories have been advanced to explain these variations, but none is as yet sufficiently substantiated.

Since a change in the water content of the organism is usually accompanied by a change in the inorganic salt balance, we have attempted to determine the effect of ingestion of large quantities of inorganic salts on the normal individual.

## EXPERIMENTAL

A normal young man, age 25 years, height 174 cm., weight 69 kilos, was placed on a low salt diet and the inorganic salt and water balances were determined. This subject had been used in previous experiments and his caloric requirement had been found to be about 3500 calories per day. To avoid monotony, three diets, each yielding 3500 calories and containing approximately the same amounts of the inorganic salts, were prepared and fed on subsequent days and repeated each 3rd day. The metabolic periods were thus 3 days in length. The subject was placed on this diet

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6 days before analyses were started. The urine was analyzed every day and the stools were mixed and analyzed for each metabolic period. 100 milli-equivalents per day of sodium, potassium, and ammonium chlorides, and sodium bicarbonate were fed in capsules in separate periods. After each feeding experiment, two or three metabolic periods were used as control periods until the subject was in balance with his diet before another salt was fed. Balances were determined for each 3 day metabolic period.

The methods used in the analysis of the urine were as follows: sodium, Butler and Tuthill (1); potassium, colorimetric, Shohl and Bennett (2); calcium, Tisdall and Kramer (3); magnesium, Briggs (4); nitrogen, macro-Kjeldahl; chlorides, Volhard-Arnold; inorganic phosphate, Fiske and Subbarow (5); inorganic sulfate, determined as barium sulfate from acidified urine; ammonia, Folin and Bell (6); and titratable acidity, Henderson and Palmer (7).

The stools were dried, weighed, and ground, and a 1 gm. sample was ashed in a silica beaker with sulfuric acid in a muffle furnace at a dull red heat.<sup>1</sup> The ash was dissolved with 10 to 25 cc. of 10 per cent hydrochloric acid, filtered into a 100 cc. volumetric flask, and the filter paper was ashed if necessary. The solution of the ash was made up to volume and aliquots taken for analysis. Sodium was determined by the method of Barber and Kolthoff (8). Potassium, calcium, and magnesium were determined by the methods used for urine. Total phosphorus was determined by the method of Fiske and Subbarow (9). Since the excretion of the chloride ion in the stool is practically negligible, no attempt was made to analyze for it.

Water balance was determined by the method of Wiley and Newburgh (10). The diets were analyzed by the methods described for stools. The chloride content of the diet was estimated from food tables (Sherman (11)).

### *Results*

The results of the experiments are given in Table I. Since the diet was very close to the maintenance requirements of the subject,

<sup>1</sup> The authors are indebted to Dr. S. L. Weight of the Department of Research Medicine, University of Pennsylvania, for suggestions concerning the method of ashing stools.

the major shifts in his water content are reflected in the change of body weight, and we have thus omitted the data on water balance. To conserve on space, the urine analyses have been reported for each experimental period and the control periods following each experiment have been summarized.

*Effect of Ingestion of Sodium Chloride*—The addition of 100 milli-equivalents of sodium chloride daily to the diet of the normal subject was accompanied by a retention of water in the first and second periods and a loss of water in the third period. This is in agreement with the results of Meyer and Cohn (12), who found that the feeding of sodium chloride to infants resulted in an increased body weight. It should be pointed out, however, that changes in body weight of the magnitude found in our experiments are often observed in subjects on a constant diet and may be of little significance in the interpretation of these data.

During the first period of sodium chloride feeding, there was a marked retention of both sodium and chlorides, about 30 per cent of the amount ingested, most of this retention occurring during the 1st day. In the second and third periods, a slight negative balance was noted for these elements. Practically all of the sodium and chlorides retained during the experimental periods were excreted in the subsequent control periods. These results tend to confirm the observations of Loeb, Atchley, Richards, Benedict, and Driscoll (13) in their study of a normal individual. Their results were complicated, however, by the ingestion of ammonium chloride at the end of the period of sodium chloride feeding without an intervening control period.

The effect of the ingestion of sodium chloride on the potassium excretion has been studied by Richards, Godden, and Husband (14) in growing pigs. They found that the increased urinary potassium was practically balanced by a decrease in the excretion of potassium in the feces. In the first period of ingestion of sodium chloride, we found a marked increase in the urinary potassium, the fecal potassium remaining constant. In the second period the fecal potassium increased and the urinary potassium fell below normal, the net result being a loss of 2 milli-equivalents of potassium. The urinary potassium returned to its normal level during the third period and the fecal excretion remained above normal. Most of the loss of potassium in the ex-



TABLE I

*Effect of Ingestion of Large Amounts of Inorganic Salts*

The diet, excluding the added salts, contained 80.5 milli-equivalents of sodium, 239 milli-equivalents of potassium, 142 milli-equivalents of calcium, and 100 milli-equivalents of magnesium per metabolic period of 3 days

	Weight	Na	K	Ca	Mg	N	Cl	P*	St	NH <sub>3</sub>	Titrat- able acid	Remarks
	gm	m-eq	m-eq	m-eq	m-eq	gm	m-eq	gm	gm	m-eq	m-eq	
Urine		163	324	21	90	46.9	230	4.48	2.60	209	174	Control period 6 days
Stool		5	62	268	138	6.3		3.44				
Balance	+97	-7	+62	-5	-28	+1.4	-20					
Urine		268	222	14	43	23.6	288	2.12	1.28	95	85	100 m-eq. NaCl daily 3 days
Stool		3	46	153	57	3.3		1.66				
Balance	+287	+109	-29	-25	0	+0.4	+117					
Urine		393	180	12	46	23.0	442	2.27	1.27	98	84	" "
Stool		3	57	133	65	3.8		1.65				
Balance	+43	-16	+2	-3	-11	+0.5	-37					
Urine		391	194	11	44	23.5	428	2.29	1.34	103	68	" "
Stool		4	58	145	72	3.8		1.65				
Balance	-175	-15	-13	-14	-16	0.0	-23					
Urine		223	350	14	82	48.3	286	4.41	2.62	211	161	Control period 6 days
Stool		5	92	249	128	6.0		3.27				
Balance	-315	-67	+36	+21	-10	+0.3	-76					
Urine		120	427	8	38	26.0	376	1.95	1.48	103	79	100 m-eq. KCl daily 3 days
Stool		2	61	131	54	2.8		1.66				
Balance...	+130	-42	+52	+3	+8	-1.5	+29					
Urine		73	559	7	38	24.1	414	2.10	1.33	96	85	" "
Stool		3	73	148	45	3.2		1.80				
Balance	-307	+4	-93	-13	+17	0.0	-9					
Urine		46	373	5	26	17.0	266	1.42	0.97	66	51	100 m-eq. KCl daily 2 days
Stool		2	40	85	34	1.8		1.04				
Balance	+157	+6	-54	+4	+7	-0.6	+4					

Urine	135	447	13	66	47 9	215	4 75	2 55	208	146	Control period 6 days
Stool	4	95	261	131	5 6	-5	3 45				
Balance	+225	-64	+10	+3	+1 1		2 63	1 30	187	99	100 m.-eq. $\text{NH}_4\text{Cl}$ daily 3 days
Urine	116	263	14	45	27 8	331	1 47				
Stool	3	48	124	42	2 7						
Balance	-215	-72	+4	+13	+1 1	+74	4 50	2 64	323	153	Control period 6 days
Urine	89	315	20	75	46 8	285	3 23				
Stool	8	130	240	118	6 8						
Balance	+547	+33	+24	+7	+1 0	-75	2 31	1 39	213	97	100 m.-eq. $\text{NH}_4\text{Cl}$ daily 3 days
Urine	140	213	19	38	29 0	259	1 61				
Stool	3	57	118	55	3 2	+146	2 62	1 53	257	101	" "
Balance	-532	-31	+5	+7	-0 7		1 79				
Urine	84	251	23	45	31 1	398					
Stool	2	40	132	66	3 2						
Balance	-238	-52	-13	-11	-2 8	+7	8 64	5 07	504	299	Control period 12 days
Urine	256	562	39	158	92 9	458	6 73				
Stool	8	178	529	259	13 6						
Balance	+1057	+216	0	-17	+2 9	-38	2 22	1 20	19	-20	100 m.-eq. $\text{NaHCO}_3$ daily 3 days
Urine	373	198	5	45	21 3	81	1 44				
Stool	2	48	115	53	3 0		2 16	1 14	18	-41	" "
Balance	+100	-8	+22	+2	+3 0	+24	1 75				
Urine	463	144	7	41	21 4	111					
Stool	2	50	133	55	3 5						
Balance	+233	+45	+2	+4	+2 4	-6	6 75	3 88	285	214	Control period 9 days
Urine	268	505	26	119	68 7	341	5 25				
Stool	6	147	380	178	11 3						
Balance	-232	+65	+20	+3	+1 9	-26					

\* Phosphorus figures for urine represent the inorganic phosphates calculated as gm. of phosphorus and the data for the stools represent the total phosphorus excretion.

† Inorganic sulfate is calculated as gm. of sulfur

perimental periods was balanced by a retention of this element during the subsequent control period.

The fecal excretion of calcium was markedly increased in the first and third periods of sodium chloride administration, resulting in negative balances for both of these periods. About one-half of the calcium lost from the body was returned during the control period. The other elements studied showed no significant changes.

*Effect of Ingestion of Potassium Chloride*—Loeb *et al.* (13) found that the administration of potassium chloride gave an abrupt increase in the pH of the urine, a decreased titratable acidity, and a decreased formation of ammonia. They attributed these changes to the relative rates of the excretion of the potassium and chloride ions, the potassium ion being excreted more readily than the chloride ion. There was a retention of both of these ions during their experiment.

In our experiments we were unable to find any significant changes in the excretion of ammonia or in the titratable acidity which could be attributed to the feeding of potassium chloride. In contrast to the results of these investigators, we found that the retention of the potassium ion in the first period of potassium chloride feeding was greater than that of the chloride ion. However, the chloride balance was approximately equal to the algebraic sum of the balances for the inorganic cations. A negative potassium balance was maintained throughout the second and third periods of ingestion and the control period, resulting in a net loss of 160 milli-equivalents of potassium during this experiment.

There was a loss of sodium from the body in the first period which was practically balanced by retentions in the second and third feeding periods and in the control period. During the first period there was a marked decrease in the excretion of inorganic phosphates and a compensatory increase in the inorganic sulfates, both approaching normal again in the second and third periods. There were no important changes in the excretion of magnesium, calcium, or nitrogen. The changes in body weight are roughly parallel to the retention or loss of potassium from the body.

*Effect of Ingestion of Ammonium Chloride*—Two feeding experiments were made with ammonium chloride; the first lasting for 3 days was followed by a control period of 6 days, and the second

lasting for 6 days was followed by a control period of 9 days. The results were much alike qualitatively. Immediately following the ingestion of ammonium chloride, there was a marked loss of sodium and potassium in the urine. Continued feeding of this salt augmented the loss of potassium in the urine with a slight decrease in fecal potassium, but the excretion of sodium returned to normal. There was a marked increase in the excretion of ammonia, which attained its maximum on the 4th day. The excretion of sodium and chloride rose to a maximum on the 3rd day and then declined in approximately equivalent amounts throughout the remainder of the feeding experiment. There was a retention of chlorides in the second experiment which was not balanced in the control period. A slight negative balance was obtained for both calcium and magnesium toward the end of the period. There was a slight increase in the excretion of inorganic phosphates and sulfates in the urine and an increase in the titratable acidity. The decline in body weight indicates a loss of not over 800 gm. of water from the body during this experiment. It is interesting to note that the increase in weight in both the control periods was greater than the loss experienced during the feeding of ammonium chloride. The results are essentially the same as those observed by Loeb *et al.* (13), Gamble *et al.* (15), and Følling (16).

*Effect of Ingestion of Sodium Bicarbonate*—Sodium bicarbonate was fed for 6 days and a subsequent 9 day interval was used as a control period. A slight gain in weight was noted during the feeding periods. There was a progressive increase in sodium elimination for 5 days, balance being established in the first period and a marked negative balance occurring in the second period. The negative balance was maintained throughout the control period. There was a marked retention of chlorides during the first period, balance being practically established in the second period. This primary retention was more than balanced during the control period. Potassium excretion was very slightly increased early in the feeding of sodium bicarbonate and later decreased so that there was a marked retention which was maintained during the control period. The excretion of inorganic phosphates was not changed to any significant degree, but the excretion of inorganic sulfates decreased. There was a decrease

in the production of ammonia and the urine became quite alkaline, as is evidenced by the negative titratable acidity. A marked retention of calcium was observed in the first period and balance was established in the second period.

#### DISCUSSION

From our results it would appear that a retention of either sodium or potassium is accompanied by a retention of water and that a loss of either is accompanied by a loss of water from the normal organism. In some experiments we found a loss of one of these elements accompanied by a retention of the other, and the effects apparently balanced each other, so that there was no change in the water balance. The results obtained from the administration of sodium bicarbonate indicate that, in equivalent quantities, a shift in potassium has a greater effect on water balance than does a change in the sodium content of the body.

The administration of sodium chloride was accompanied by a loss of potassium, while the feeding of sodium bicarbonate caused a retention of potassium. These results substantiate the observations of others that the anion is of more importance than the cation.

The ingestion of potassium chloride caused an increased excretion of sodium, potassium, and chlorides; the increase in the latter being approximately equal to the sum of the increases of the two bases. This, together with the fact that there was no change in the titratable acidity of the urine, is not in conformity with the findings of Loeb *et al.* (13), who noted a more rapid increase in the excretion of potassium than in the excretion of chlorides. They conclude that the potassium ion was absorbed and excreted more rapidly than the chloride ion. We have found no evidence to support this hypothesis. The discrepancy in the results may be due to the differences in the amounts of potassium chloride administered.

Certain differences in the effects of the administration of these salts are interesting, but their significance is yet to be determined. The feeding of sodium chloride resulted in a retention of sodium. The administration of potassium chloride was accompanied by a loss in potassium from the body. And sodium bicarbonate caused a loss of sodium and a retention of potassium. No relationship

could be established between the excretion of the various bases. It appears, however, that the excretion of chlorides parallels the output of sodium more closely than that of any other base.

#### SUMMARY

1. Sodium, potassium, and ammonium chlorides, and sodium bicarbonate were fed in equivalent quantities to a normal man on a salt-poor, maintenance diet.

2. Sodium chloride administration was accompanied by a negative potassium balance, an early sodium and chloride retention followed by an increased excretion of both, an increased excretion of both urinary and fecal calcium, and slight changes in body weight.

3. Feeding of potassium chloride caused an increase in the excretion of sodium and potassium, resulting in a negative balance for each of these elements, a decrease in the inorganic phosphates, an increase in inorganic sulfates, and no marked change in body weight.

4. Ammonium chloride ingestion was accompanied by negative balances in sodium and potassium, the latter being quite marked, slight negative balances for calcium and magnesium, and an increased ammonia formation and titratable acidity. The body weight declined during the feeding periods, indicating a loss of body water, and in the control periods the gain in weight more than balanced this loss.

5. Sodium bicarbonate caused a slight retention of water, a negative sodium balance, a positive potassium balance, a slight decrease in chloride excretion, and a marked decrease in ammonia formation.

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## THE INORGANIC SALT BALANCE DURING DEHYDRATION AND RECOVERY\*

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The inorganic bases of the body fluids, especially sodium and potassium, are quite individual in their distribution. A consideration of data obtained by Katz (1), Gamble, Ross, and Tisdall (2), Kramer and Tisdall (3), and Loeb, Atchley, and Palmer (4), indicates that sodium is the predominant base of blood serum and edema fluids, and that potassium is in excess in blood cells and muscle. Since edema fluids are comparable in their content of inorganic bases to the blood serum from which they originate, it seems safe to assume that the interstitial fluids, which bathe the muscle cells and undoubtedly have the same source as edema fluids, also resemble blood serum in composition. Because of the characteristic distribution of these elements it was thought that a careful consideration of the foregoing data might indicate the source of the excess water excreted in dehydration, since a loss of water from any part of the body must be accompanied by a loss of dissolved solids to prevent any change in the osmotic pressure of the remaining fluid.

This investigation was made in conjunction with some studies on dehydration being performed by Newburgh and Johnston (5), and the authors wish to express their gratitude to these investigators for their cooperation and for the opportunity to make this study.

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## EXPERIMENTAL

A normal man, age 23 years, height 180 cm., weight 59 kilos, was used as the subject for this study. He was fed a diet which approximated his caloric requirement and which could be prepared from day to day with little variation in the water content. The available water, except for slight daily variations in preformed water and water of oxidation, could be varied at will by adjusting the water intake. The experiment is divided into several periods of varying lengths, the daily water intake being practically constant for each period.

The urine was collected under oil and analyzed daily, by the following methods: sodium, Butler and Tuthill (6); potassium, colorimetric, Shohl and Bennett (7); calcium, Tisdall and Kramer (8); magnesium, Briggs (9); total base, Stadie and Ross (10); nitrogen, macro-Kjeldahl; chorides, Volhard-Arnold; inorganic phosphate, Fiske and Subbarow (11); inorganic sulfates, determined as barium sulfate from acidified urine; ammonia, Folin and Bell (12); titratable acidity, Henderson and Palmer (13); pH, Myers and Muntwyler (14).

The stools were dried, weighed, and ground and a 1 gm. sample ashed in a silica beaker with sulfuric acid. The ash was dissolved with 10 to 25 cc. of 10 per cent hydrochloric acid, filtered into a 100 cc. volumetric flask, and the filter paper ashed if necessary. The solution of the ash was made up to volume and aliquots taken for analysis. Sodium was determined by the method of Barber and Kolthoff (15). The other determinations were made by the methods used for urine.

Water balance was determined by the method of Wiley and Newburgh (16). The diets were analyzed by the methods applied to stools. The chloride content of the diet was calculated from food tables (Sherman (17)).

*Results*

The results of this study are presented in Table I. The sodium and chloride contents of the stools were found to be negligible and are not included in Table I. The stool analyses are reported for each period rather than daily.

The diet was found to contain the following amounts of inorganic

ions per day: sodium, 139 milli-equivalents; potassium, 54.2 milli-equivalents; calcium, 22 milli-equivalents; magnesium, 16.6 milli-equivalents; and chlorides, 140 milli-equivalents.

After the subject had been fed the experimental diet for several days, the examination of the urine and stool was started. Period I was a control period in which the subject was allowed water *ad libitum*. There was some shift in water balance of about the magnitude normally encountered in studies of this kind. The sodium excretion in the urine indicated that the subject was not yet in sodium balance, since the lowest figure occurred with the greatest loss of body water. There was also a slight negative potassium balance during this period.

In Period II, the water intake was limited so that the available water, including preformed and oxidation water, would amount to about 1400 gm. per day. The total loss of body water for this period was 769 gm., 663 gm. being lost on the 1st day. The sodium balance was about the same as found in the control period. There was a slight increased loss of potassium over the control period, divided equally between the urine and stool. The chloride excretion on the 1st day of the period was high and dropped below normal on the last 2 days. The loss of inorganic salts was not as great as had been anticipated for a period of dehydration of this extent, but may be accounted for by the failure of the subject to attain sodium and chloride balance before the experiment began.

In Period III, the water intake was increased for 2 days so that the available water would amount to about 2200 gm. per day. There was a net water retention of 275 gm. accompanied by a retention of sodium and chlorides. There was a marked loss of calcium and a slight loss of potassium and magnesium.

For 1 day, Period IV, the available water was reduced to 1411 gm. and for 6 days, Period V, it amounted to about 1185 gm. per day. In Period IV there was a loss of 219 gm. of body water accompanied by a loss of potassium, calcium, and magnesium. The figures for sodium and chlorides indicate that these ions had approximately attained balance. The loss of 1939 gm. of body water in Period V was accompanied by a marked loss of sodium, potassium, and chlorides and a slight loss of calcium, magnesium, and inorganic sulfates.

The water intake in Period VI was adjusted so that the subject

TABLE I  
*Inorganic Salt Balance during Dehydration*

Period No	Date	Weight kg.	Na m.-eq	K m.-eq	Ca m.-eq	Mg m.-eq	NH <sub>3</sub> m.-eq	pH	Titratable acid m.-eq	Cl m.-eq	Inor- ganic SO <sub>4</sub> m.-eq	Inor- ganic PO <sub>4</sub> m.-eq	H <sub>2</sub> O in gm	H <sub>2</sub> O out gm	H <sub>2</sub> O balance	
															+	-
I	Oct. 26	59 208	107	51 8	10 8	10 7	32 6	6 4	22 9	120	29 2	75 1	2295	2583	gm.	288
	" 27	59 238	120	51 1	11 0	11 5	29 1	6 3	22 9	130	33 6	78 1	2102	2098	4	
	" 28	59 196	132	50 8	10 5	11 6	23 9	6 5	26 4	127	31 8	74 4	2427	2459		19
Stool Balance			+58	25 6 -16 7	33 3 +0 4	18 3 +2 4				+43						303
II	Oct. 29	58 552	132	55 3	9 0	8 2	25 3	6 2	22 1	148	32 2	73 7	1402	2065		663
	" 30	58 469	110	53 4	9 5	10 8	24 3	5 8	25 0	122	32 6	77 4	1411	1481		70
	" 31	58 455	120	50 8	11 0	8 6	22 3	6 1	25 7	124	34 8	88 2	1408	1444		36
Stool Balance			+55	30 8 -27 7	43 8 +7 3	18 5 -3 7				+26						769
III	Nov. 1	58 927	86	38 7	9 5	10 9	26 3	6 1	23 6	78	31 4	76 4	2197	1706	491	216
	" 2	58 732	138	50 6	12 0	11 1	26 8	6 5	28 6	133	35 2	88 4	2142	2358		
Stool Balance			+53	22 5 -3 4	34 8 -12 3	16 2 -5 0				+69					275	
IV	Nov. 3	58 492	138	50 4	14 8	11 1	24 0	6 2	28 6	141	35 2	76 8	1411	1630		219
	Stool Balance		+1	12 5 -8 7	17 8 -10 6	8 1 -2 6				+1						219

V	Nov	4	57 967	166	54 6	14 2	15 2	23 8	6 1	27 1	177	38 8	73 7	1189	1704	515
	"	5	57 642	143	40 6	14 5	13 8	24 2	6 0	27 1	170	35 0	59 7	1173	1504	331
	"	6	57 212	163	50 9	16 0	11 5	20 7	6 1	25 7	176	39 2	65 8	1186	1612	426
	"	7	56 991	144	49 4	11 7	12 8	16 9	6 0	27 1	150	40 2	74 1	1193	1433	240
	"	8	56 769	152	63 9	10 0	12 9	23 6	6 1	28 6	158	43 4	81 4	1182	1421	239
	"	9	56 589	137	56 2	9 5	8 6	18 0	6 0	31 4	153	40 2	77 0	1181	1369	188
Stool Balance				-70	70 8 -61 2	62 4 -6 3	37 2 -2 4				-144					1939
VI	Nov	10	56 514	159	64 1	11 5	13 1	19 5	6 1	30 7	158	46 6	93 2	1404	1443	39
	"	11	56 409	123	60 2	11 7	15 0	22 4	5 9	33 6	135	45 4	93 2	1413	1483	70
	"	12	56 504	127	56 0	10 5	13 4	17 3	6 0	32 1	127	43 6	81 0	1406	1328	78
	"	13	56 559	122	53 8	10 5	9 0	22 5	5 8	35 0	128	43 0	90 0	1358	1320	38
	"	14	56 606	147	56 4	11 1	8 6	23 5	6 0	33 6	148	46 8	102 0	1422	1388	34
Stool Balance				+17	36 1 -55 6	61 6 -6 9	26 6 -2 7				+4					41
VII	Nov.	15	56 919	129	41 3	10 5	8 9	23 3	5 9	32 9	122	42 4	90 0	1622	1327	295
	"	16	57 104	167	51 7	11 8	11 9	23 3	6 1	32 1	164	46 0	99 8	1616	1418	198
	"	17	57 392	152	45 9	11 8	13 4	25 6	6 1	37 9	147	45 0	100 0	1614	1340	274
	"	18	57 667	132	33 0	13 7	11 5	24 6	6 0	35 7	125	44 0	89 7	1623	1366	257
	"	19	57 922	133	35 3	13 8	11 7	28 0	6 0	29 3	131	42 2	86 0	1618	1381	237
	"	20	58 132	139	29 7	14 3	12 2	27 0	6 1	32 9	134	39 2	83 6	1594	1366	228
Stool Balance				144	32 1	13 3	13 4	25 5	6 1	32 9	141	39 0	89 5	1625	1420	205
Stool Balance				-25	42 7 +67 7	89 6 -24 8	35 7 -2 5				+14					1694

TABLE I—Concluded

Period No	Date	Weight <i>kg</i>	Na	K	Ca	Mg	NH <sub>3</sub>	pH	Titratable acid	Cl	Inorganic SO <sub>4</sub>	Inorganic PO <sub>4</sub>	H <sub>2</sub> O in <i>gm</i>	H <sub>2</sub> O out <i>gm</i>	H <sub>2</sub> O balance	
			<i>m-eq</i>	<i>m-eq</i>	<i>m-eq</i>	<i>m-eq</i>	<i>m-eq</i>		<i>m-eq</i>	<i>m-eq</i>	<i>m-eq</i>	<i>m-eq</i>	<i>gm</i>	<i>gm</i>	+	-
VIII	Nov 22	58 737	128	34 0	15 7	12 7	33 3	6 0	27 1	135	37 4	79 4	1817	1454	363	
	" 23	58 882	141	28 3	16 3	10 2	32 3	6 2	28 6	137	34 6	81 0	1803	1610	193	
	" 24	58 970	138	28 3	15 2	12 5	30 7	6 3	27 9	140	31 8	75 5	1829	1763	66	
	" 25	58 914	172	35 9	15 2	11 0	26 5	6 2	30 0	176	35 8	80 6	1829	1894		55
	" 26	58 880	183	40 1	15 5	9 4	28 9	6 2	29 3	187	31 8	82 8	1832	1887		55
	" 27	58 912	129	38 2	13 5	11 8	30 4	6 2	27 1	135	31 8	79 4	1833	1762	71	
Stool Balance			-57	+83 8	+20 4	+1 4				-70					583	

could approximately maintain his body weight. There was a net retention of 41 gm. of water in 5 days. This was accompanied by a loss of potassium, calcium, and magnesium and a retention of sodium. There was a very marked increase in the excretion of inorganic phosphates and a slight increase in inorganic sulfates.

The available water in Period VII was increased to about 1620 gm. per day. There was a fairly constant daily retention of water which amounted to 1694 gm. for the entire period. This retention of water was accompanied by a retention of potassium, and a loss of sodium and calcium. There was a slight retention of chlorides and an increased excretion of inorganic sulfates. The output of inorganic phosphate gradually decreased but did not reach normal.

In Period VIII, the available water was increased to about 1820 gm. per day for 6 days. There was a marked water retention in the first 2 days after which water balance was established. There was a loss of sodium and chlorides in this period and a retention of potassium and calcium. The excretion of inorganic sulfates and phosphates returned to normal.

Dehydration was accompanied by a decreased excretion of ammonia and a decrease in the urinary pH. These changes were not of enough magnitude to permit any interpretation of their significance. There was an increase in the titratable acidity, which paralleled the increased excretion of inorganic phosphates.

#### DISCUSSION

The observations in Periods I, II, III, and IV indicate that a slight shift in body water can be obtained without any significant change in the amount of inorganic salts excreted. The maximum shift in body water in these periods was about 1.5 per cent of the body weight. These results may indicate an ability of the normal organism to concentrate body fluids to a slight extent.

The continued restriction of water intake resulted in a water loss by the end of Period V equivalent to 5 per cent of the body weight. The greatest excretion of sodium in Period V occurred on the 1st day, and the smallest excretion was obtained on the last day. In contrast to these results, the excretion of potassium was more marked at the end of the period than at the beginning. We are led to conclude from these data that water was first lost from the blood serum and interstitial fluids and later was drawn from

the cells. This is in accord with the findings of Underhill and Errico (18).

In Period VI, where there was little change in the water content of the subject, a slight retention of sodium and a marked loss of potassium occurred. The loss of potassium was over 3 times the sodium retention. These facts support the evidence obtained in early periods, indicating that some concentration of the body fluids is possible. It also shows that in some conditions, the converse is true; namely, that the body can lose inorganic salts without an accompanying loss of water. This change of base which took place under conditions of water balance, also lends more support to the evidence obtained by Underhill and Errico (18), on the primary concentration of the blood during dehydration. Evidently there was a shift of water from the cells to the blood serum or the interstitial fluids, resulting in a demand for sodium by these fluids and the liberation of an excess of potassium to be excreted.

The excessive excretion of inorganic phosphates and sulfates is interesting in view of the statement of Howland and Marriott (19), that phosphates are more likely to be retained in the blood than chlorides. The average inorganic phosphate excretion in Period V is lower than that of Period I, indicating that there was some retention during dehydration. The extra output of phosphate when water balance has been established cannot be accounted for on the basis of this previous retention, since the excess excretion in Period VI was about 4 times the retention in Period V. This may indicate a loss of phosphates from the cells as a result of the continued dehydration. Unfortunately, we have no data on the phosphate content of the blood cells to verify this hypothesis.

There is no evidence in the data to show that the kidneys were not able to excrete all of the material presented to them in Period V, since an increase of over 200 gm. in available water in Period VI was accompanied by a reduction in the excretion of inorganic substances. It is also interesting to note that in the data presented on this study by Newburgh and Johnston (5) there was no decrease in insensible water due to dehydration. This does not agree with the findings of Atchley *et al.* (20), who state that "there appeared to be a decrease in water loss through the skin during the acidosis and recovery periods." It is true that their subjects were dehydrated, but it is difficult to understand how

they could assume a decrease in the loss of water through the skin, since they made no attempt to quantitatively estimate this loss.

The marked retention of potassium and the loss of sodium in the recovery phase, Periods VII and VIII, indicate that most of the water retained was stored in the cells. We must assume from this data that, while the blood may be first to give up water during dehydration, the ultimate effect is a restoration of the blood at the expense of the cellular water.

In these experiments it was found that the titratable acidity of the urine paralleled the excretion of inorganic phosphates and the excretion of inorganic sulfates paralleled the urinary nitrogen.

#### SUMMARY

A normal man on a constant diet was dehydrated to the extent of 5 per cent of his body weight, and the inorganic salt and water balances determined, for both the dehydration and recovery periods. The results of this study are as follows:

1. A slight dehydration, about 1.5 per cent of the body weight can be accomplished without any great disturbance of the salt balance, due probably to the ability of the organism to concentrate its fluids to some extent.

2. More marked dehydration is accompanied by a loss of body potassium, sodium, and chlorides. The results indicate that the blood serum is first to respond to water deprivation. However, the continued depletion of body potassium evidently means that there is an accompanying loss of cellular water.

3. When further depletion of endogenous water was avoided, there was a continued loss of body potassium and inorganic phosphates and a retention of sodium. Since this occurred without a loss of body water, it appears to be true that a shift of water from cells to blood serum and interstitial fluids was taking place.

4. Subsequent administration of water in amounts sufficient to permit a restoration of the original water content of the body was accompanied by a loss of sodium and a retention of potassium, which indicates that most of the water retained was being stored in the cells. It must also be assumed from these data that most of the water lost during dehydration was ultimately drawn from the cells.



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## PHOSPHATASE STUDIES

### II. DETERMINATION OF SERUM PHOSPHATASE.\* FACTORS INFLUENCING THE ACCURACY OF THE DETERMINATION

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(Received for publication, March 21, 1933)

We found Kay's method (2) unsatisfactory for our purposes in studies of experimental and clinical variations of plasma phosphatase. We abandoned it with reluctance, for Kay had reported important clinical results in terms of his method (3). The 48 hour incubation period used by Kay was a source of the greatest difficulties: variable retardation by the products of hydrolysis occurred in analyses of plasmas or sera high in phosphatase; the limits of the method were frequently exceeded.

Shorter periods of incubation were essential, particularly in studies requiring repeated phosphatase determinations. By using veronal to maintain the pH of our reaction mixture at about 8.6, and by using Kuttner and Lichtenstein's sensitive method in a slightly modified form (4-7) in the determination of inorganic phosphate, we were enabled to decrease the incubation period to 2 hours, and the volume of oxalated plasma (*twice centrifuged to avoid filtration*) to 1 to 3 cc.; our phosphatase unit was similar to Levene and Dillon's (8). We described this method briefly (9) and discussed it in the presentation of a study of phosphatase variations in experimental hyperparathyroidism (10) at the meeting of the American Society of Biological Chemists in April, 1931.

"After seeing the improved Kuttner-Cohen . . . procedure as demonstrated by Youngburg" (11) at the same meeting, Jenner

\* This method was demonstrated by Bodansky and Chandler at the Twenty-sixth annual meeting of the American Society of Biological Chemists at Philadelphia, April, 1932, and briefly outlined in the presentation of a study of experimental variations of serum phosphatase (1).

and Kay "decided to adapt this very delicate modification of the Denigès reaction" in a new plasma phosphatase method (12) in which a glycine-NaOH-NaCl buffer was employed. This method was intended specifically for the clinical laboratory. Jenner and Kay offered no estimate of the accuracy of their method; however, a comparison of the results which they obtained by the new and the old methods shows great discrepancies, and in the higher range *their new method gives even lower values* (see p. 103).

In the meantime, in the continued use of our method we had added to its accuracy and convenience by the use of serum instead of oxalated plasma (13), by corrections for analytical errors in the presence of glycerophosphate and trichloroacetic acid (14), by the use of a combined solution of buffer and substrate of proved keeping quality, and by the reduction of the time of incubation to 1 hour and less. The calculations were simplified by changing the final dilution of the serum in the "total inorganic P filtrate" from 16 to 20 (at the suggestion of Dr. J. P. Chandler of this laboratory). The error of our phosphatase determination is about 2 to 3 per cent in the range of pathological values (see below). We have tested our method in over a thousand analyses and in a great variety of clinical and experimental material (1, 10, 13, 15-18). The details follow.

#### *Determination of Serum Phosphatase*

**Buffered Substrate**—Dissolve 2.5 gm. of sodium glycerophosphate<sup>1</sup> and 2.12 gm. of monosodium diethylbarbiturate (Merck's barbital-sodium United States Pharmacopœia X) in a 500 cc. volumetric flask. We preserve the substrate in the refrigerator in glass-stoppered bottles (100 or 250 cc.) under a layer of washed petroleum ether (b. p. 30-36°) about 3 cm. thick.

**10 Per Cent Trichloroacetic Acid Solution**—J. T. Baker, C.P. Analyzed.

**Solutions and Reagents Required for Serum Inorganic Phosphate Determination (14)**—Standard phosphate solution (5 cc. equivalent to 0.02 mg. of P); freshly prepared acid-molybdate reagent; freshly prepared dilute stannous chloride solution (0.3 per cent).

<sup>1</sup> We have been using sodium  $\beta$ -glycerophosphate, Boots Pure Drug Company, Ltd., Nottingham, England, recommended by Kay (2). Eastman Kodak sodium glycerophosphate (Eastman Kodak Research Laboratories) gives similar results.

*Preparation of Filtrates for Analysis*—Measure 10 cc. of substrate (equivalent to about 5 mg. of P) into a test-tube 18 or 20 mm.  $\times$  150 mm.,<sup>2</sup> avoiding aeration of substrate; place it in a water bath at 37° for a few minutes; add 1 cc. of centrifuged serum; mix by a single inversion and replace in the water bath for exactly 1 hour.<sup>3</sup> Remove, cool immediately in ice water, add 9 cc. of 10 per cent trichloroacetic acid, mix, and filter after a few minutes through Whatman filter paper No. 44 (11 cm.), or similar paper of low ash content (*"total inorganic P filtrate,"* each cc. equivalent to 0.05 cc. of serum). Use 1 cc. of serum (1.5 or 2 cc., if the inorganic phosphorus is expected to be less than 3 mg.), precipitated with 9 volumes of 5 per cent trichloroacetic acid, to obtain the filtrate for an *inorganic serum phosphate analysis* (14).<sup>4</sup> The filtrates may be saved in the refrigerator until analysis; *analyses performed immediately and after several days yield excellent checks.*

Half quantities may be used; high phosphatase sera may be incubated  $\frac{1}{2}$  or  $\frac{1}{4}$  hour (see p. 100).

*Analysis and Calculations*—Aliquots containing 0.012 to 0.036 mg. of P may be used. The determination of serum inorganic phosphate and of total inorganic phosphate after incubation with substrate has been described (14). In calculating our results, we enter (a) the aliquot volume,  $V$ , (b) colorimetric readings (the 0.02 mg. standard being set at 20 mm.), (c) the corresponding aliquot values,  $T$ , in mg. of inorganic P, as given in a published table

<sup>2</sup> We prefer glass-stoppered test-tubes, although rubber stoppers may be employed with ordinary test-tubes. The glass-stoppered test-tubes were made for us by the Scientific Glass Apparatus Company, Bloomfield, New Jersey (interchangeable stoppers), and by Eimer and Amend, New York.

<sup>3</sup> When an incubator is used, a beaker of warm water should be used for preheating the substrates to 37°. An inexpensive and very satisfactory constant temperature water bath may be constructed by following the general directions of Findlay (19).

<sup>4</sup> The quantity of serum required for the inorganic phosphate analysis may be reduced by employing 2 cc. of filtrate, 2 cc. of acid-molybdate reagent, and 1 cc. of 0.15 per cent stannous chloride solution (total volume 5 cc.). Furthermore, while we make our most accurate comparisons against the 0.02 mg. standard, an analyst capable of reading as accurately against a 0.01 mg. standard may halve the necessary quantities of serum in both the serum inorganic phosphate and the "total inorganic phosphate" determinations.

of inorganic P values corrected for the deviation from Beer's law (14), (d) corrections for trichloroacetic acid or for trichloroacetic acid *plus* glycerophosphate contained in an aliquot (14), (e) the corrected aliquot value, *C*.

2000 *C/V* = mg. total inorganic P, after incubation with substrate, per 100 cc. serum (20 being the dilution of serum in the "total inorganic P filtrate")

1000 *C/V* = mg. inorganic P per 100 cc. serum (10 being the dilution of serum in "serum inorganic P filtrate")

The total inorganic P after incubation *minus* the serum inorganic P *equals* the liberated inorganic P. The results are stated in units per 100 cc. of serum, a unit of phosphatase activity being defined as *equivalent to 1 mg. of P liberated from a sodium glycerophosphate substrate as the phosphate ion during the 1st hour, at pH 8.6 and at 37°*.

*Calculated Maximum Analytical Error*—Assuming an error of 2 per cent in the determination of inorganic P (14), the maximum possible error in the phosphatase determination would equal  $2 + 4$  (serum inorganic P/liberated P) per cent. This formula has been used to calculate the maximum possible errors over the known range of serum inorganic phosphate and serum phosphatase: in *sera high in phosphatase* the calculated maximum error of the phosphatase determination obviously approaches that of the inorganic phosphate analysis; in *sera low in phosphatase* (1 to 4 units per 100 cc.) the calculated maximum errors would be respectively 14 and 5 per cent when the inorganic phosphate equals 3.0 (incubation period of 1 hour). Such errors would be of no *clinical* significance.

*Actual Analytical Errors*—However, the calculated possible maximum errors have been rarely approached in our actual experience. Numerous duplicate analyses of sera high in phosphatase, the record of which must be omitted here, yielded results agreeing within 2 per cent or less, while sera low in phosphatase agreed within 5 per cent or less. (Tables I and II illustrate, incidentally, the close agreement between separate analyses of the same serum.) When greater accuracy was desired, we incubated sera low in phosphatase for 2 hours. The 2 hour results were converted into standard terms by the use of the conversion factor 0.55 (see p. 100).

*Dilution Checks*—The accuracy of the method should not be checked by diluting a serum of known high phosphatase activity with *water*, for the diminished buffer effect of the serum after dilution affects the pH of the reaction mixture. When sera high in phosphatase were diluted with *sera low in phosphatase* the activity of the mixtures in eleven determinations equaled the average of the activities of the ingredients, within the narrow limits of analytical error.

*Factors Influencing Accuracy of the Determination. Phosphatase Activity Ratios*

In order to estimate the influence of the several factors, the phosphatase activity under other than standard conditions was compared with that under standard conditions. The ratio of the former to the latter was termed phosphatase activity ratio. To save space, our data must be presented in summary form.

*Effect of Potassium Oxalate*—The average phosphatase activity ratio of oxalated plasma in thirty-seven consecutive analyses was about 0.90. The lowered phosphatase activity is possibly due to the increase of osmotic pressure by oxalate and the consequent dilution of the plasma. The *process of clotting* does not contribute to the relatively higher phosphatase content of the serum: *heparin* plasma showed an average activity ratio in twelve consecutive determinations of 0.97, the average activity ratio in parallel analyses of oxalated plasma being 0.89; serum obtained by allowing *hemophiliac plasma* to clot showed an average activity ratio of 1.02 when compared with the serum that separated in the usual manner (four specimens), parallel analyses of oxalated plasma showing an average phosphatase activity ratio of 0.90.

The handling of the clot, even when deliberately violent, raised the serum phosphatase slightly, if at all (highest activity ratio, 1.05).

Slight hemolysis (deliberately produced) had no appreciable effect.

*Choice of Substrate*—We have compared sodium  $\beta$ -glycerophosphate with Eastman Kodak glycerophosphate<sup>1</sup> in forty-one parallel determinations (3 to 130 units per 100 cc.), and have obtained with the latter a minimum activity ratio of 0.93, a maximum ratio of 1.07, and an average of 1.00. While it seems that

the Eastman Kodak preparations are similar in behavior and composition the Boots product is to be preferred as a substance of *known* composition.

*pH of Substrate*—When substrate, buffer, and serum are mixed in the prescribed ratios, the pH of the reaction mixture is about 8.6. As has been pointed out by Levene and Dillon (8), the products of hydrolysis of sodium glycerophosphate at this pH cause no change in it. Sodium diethylbarbiturate has been shown (20) to be a satisfactory buffer at this pH. Thus a constant pH was assured during the course of the reaction. Its importance has been pointed out by Kay (2). No change of pH could be demonstrated colorimetrically when incubation of our reaction mixture continued for 72 hours; we therefore concluded that no change of pH occurred during 1 or 2 hours of incubation, and that no pH controls were necessary.

Jenner and Kay criticized the use of sodium diethylbarbiturate. They stated that it was much more expensive than glycine and that "its use [was] occasionally complicated by the precipitation of di-ethyl barbituric acid when acid filtrates containing veronal were allowed to stand." Neither assertion had been found true in our experience. It is possible that Jenner and Kay used an unnecessarily expensive preparation of sodium diethylbarbiturate, and in a higher concentration than we employed.

A significant error (up to 40 to 50 per cent) may be caused by the *absorption of carbon dioxide* by alkaline substrates. The keeping quality (maintenance of the pH) may be checked more accurately by the phosphatase activity ratios of preserved and fresh substrates than by colorimetric comparison. When the substrate was covered with 3 cm. of petroleum ether (b. p. 30—36°) in small bottles and saved for 2 months in the refrigerator, the activity ratios were  $1.0 \pm 0.03$ .

*Thermolability and Incubation Temperature*—An hour or more may elapse between the collection of the blood specimen and the addition of the separated serum to the substrate; this delay has no significant effect upon the serum phosphatase. In sera remaining at room temperature for as long as 6 hours a *slight increase* of phosphatase activity was indicated.

In a study of thermolability of serum phosphatase at pH 8.6, we found that, while serum phosphatase inactivation was less at

30°, 25°, and at 0° than at 37°, the rates of hydrolysis at the lower temperatures decreased to a relatively greater extent. It became obvious that the rate of hydrolysis rather than the rate of inactivation must be considered in choosing the temperature of incubation in the *serum phosphatase* analysis; 37° was therefore chosen.

*Paradoxical Increase of Phosphatase Activity in Preserved Serum*—When we compared the serum phosphatase immediately after separation and after 24 hours in the refrigerator, *we found increased activity in all the preserved specimens* (16). We eliminated the possibility that yeasts or other microorganisms had caused or contributed to this effect in a series of analyses in which *toluene controls* were included; fresh substrates were used to secure the greatest accuracy in the quantitative estimation of the effect. The average increase of phosphatase activity after 24 hours in the refrigerator (forty-five comparisons) was about 10 per cent; the analyses of sera preserved with and without toluene differed no more than any two analyses of the same serum (by about 3 to 5 per cent). The increase of serum phosphatase activity continues for a variable length of time at 0°; the expected decrease in phosphatase activity follows, sometimes after 3 to 5 days (18).

A similar effect was obtained after the serum was kept at 37° for 1, 2, 4, and 6 hours (six specimens); after 4 to 6 hours the increase in phosphatase activity amounted to about 15 to 20 per cent.<sup>5</sup>

After thorough separation of the serum it is therefore desirable to keep it no longer than about 1 hour at room temperature or 3 to 4 hours in the refrigerator. On the other hand, *clinically useful* determinations may be obtained on serum kept in the refrigerator with a drop of toluene for 24 or 48 hours. While the results may be about 10 to 20 per cent high, the clinical interpretation would not be affected.

*Effect of Inorganic Phosphate on Rate of Hydrolysis*—The products of hydrolysis do not cause an *appreciable* error until a total of about 0.6 mg. of inorganic phosphorus is reached (equivalent to about 60 mg. per 100 cc.); that is, until after about 10 to 12 per cent of the substrate has been hydrolyzed.

<sup>5</sup> A similar "activation" of *phosphatase* after about  $\frac{1}{2}$  hour at 40–50° was observed by Euler and Ohlsén (21).



*Incubation Period*—Before we discovered the analytical errors caused by trichloroacetic acid and glycerophosphate, we concluded that, "The reaction is retarded so little during the first 2 hours that the initial velocity may be calculated . . . without substantial error" (9). We have applied correction factors to our old data and have verified the course of the reaction during the first 6 hours in new studies. Numerous routine analyses in which we incubated serum-substrate mixtures for 1 and 2 hours indicated a conversion factor of 0.55, with a possible error of  $\pm 0.02$ . We checked these results in a comparison of the analyses of fourteen sera, using freshly prepared substrates in order to avoid any errors that might have been caused in the routine analyses by incubation at even slightly differing pH. The conversion factor yielded by this series was 0.545.

We have extended our tests to both longer and shorter incubation periods. If the amount liberated within the 1st hour is taken as 1, the following relations obtain, *provided the total inorganic phosphorus does not exceed the equivalent of 50 to 60 mg. per 100 cc.*

Incubation period, min	10	15	20	30	(60)	120	180	240	360
Ratio of liberated P	0 215	0 30	0 39	0 55	(1 0)	1 82	2 57	3 30	4 70
Conversion factor.	4 70	3 30	2 57	1 82	(1 0)	0 55	0 39	0 30	0 215

*The validity of our conversion factors has been established in numerous tests over the entire range of phosphatase values.*

The conversion factor 0.55, applied to results obtained after 2 hours incubation of sera low in phosphatase, helps minimize the error, although analyses after 1 hour's incubation are sufficiently accurate for clinical diagnosis, as shown in Table I. Sera high in phosphatase should be incubated, for most accurate results, for 15 or 30 minutes, in order to avoid retardation of hydrolysis. (We have also used 10 and 20 minute periods.) The above conversion factors may be used to express the results in standard terms. Table II shows, first, the agreement of the results after 1 hour's incubation with the results obtained during the shorter intervals, when phosphatase activity did not exceed 50 to 60 units, and, secondly, the close agreement of the results calculated from the

TABLE I

*Phosphatase Analyses of Sera Low in Phosphatase after 2 Hours and 1 Hour's Incubation*

2 hrs		1 hr	2 hrs		1 hr
Found	Converted	Found	Found	Converted	Found
mg per 100 cc	units per 100 cc	units per 100 cc	mg per 100 cc	units per 100 cc	units per 100 cc
2 6	1 4	1 4	5 5	3 0	3 1
2 7	1 5	1 4	5.7	3 1	3 4
2 7	1 5	1 5	5 8	3.2	3 0
3 3	1 8	1 8	6 1	3 4	3 1
4 4	2 4	2 6	6 3	3 5	3 4
4 5	2 5	2 7	6 6	3 6	3 6
5 2	2 9	2 8	6 8	3 7	3 5
5 3	2 9	3 1	6 8	3 7	3 6

TABLE II

*Phosphatase Analyses of Sera High in Phosphatase after Various Incubation Periods*

. The results are stated in terms of mg of inorganic phosphorus liberated per 100 cc. of serum

Incubation period									
15 min		30 min		1 hr	15 min		30 min		1 hr
Found	Converted	Found	Converted	Found	Found	Converted	Found	Converted	Found
2 8	9 2	5 3	9 6	10 0			30 8	56 0	52 4
		7 6	13 8	14 2	19 5	<b>64.4</b>	35 3	<b>64.3</b>	58 9
		7 9	14 4	14 5	29 4	<b>97.0</b>	52 9	<b>96.3</b>	89 5
5 2	17 2	9 0	16 4	16 7			53 7	<b>97.8</b>	87 9
		9 0	16 4	17 1	30 1	<b>99.3</b>	53 6	<b>97.6</b>	89 1
		9 8	17 8	18 5	30 4	<b>100.0</b>	53 0	<b>96.5</b>	
7 3	24 1	13 0	23 7	23 6	32 4	<b>107.0</b>	58 4	<b>106.0</b>	
8 2	27 0	15 3	27 9	27 3			58 7	<b>107.0*</b>	100 0
9 1	30 0	17 0	30 9	30 1			60 9	<b>111.0†</b>	
9 7	32 0	17 3	31 5	30 6	38 8	<b>128.0</b>	68 9	125 0	110 0
		17 6	32 0	33 1	38 9	<b>128.0</b>	65 0	118 0	106 0
13 0	42 9	25 4	46 2	43 1	39 1	<b>129.0</b>	65 7	120 0	112 0

The figures in bold-faced type represent values preferred, having been calculated from figures obtained after hydrolysis of less than about 12 per cent of the substrate.

\* Also converted values of 107 units after 10 minutes and 104 units after 20 minutes.

† Also a converted value of 113 units after 20 minutes.

several shorter periods in analyses of sera of higher phosphatase activity. When 1 hour's incubation is used, the error may be minimized by applying a correction of 10 to 15 per cent when the phosphatase activity is 100 to 150 units per 100 cc.; the results are valid for clinical purposes.

*Phosphatase Determinations on Small Volumes of Serum*—Fractional quantities of serum high in phosphatase and substrate may be used in the reaction mixture. The substrate should then be measured into the test-tube with the tip of the pipette reaching below the surface of a 1 cm. layer of petroleum ether to reduce errors due to carbon dioxide absorption. The calculations are modified in accordance with the final dilution.

#### DISCUSSION

The increased accuracy of the phosphatase determination adds certainty to our observation that the serum phosphatase in normal subjects, in experimental controls, and in an "unvarying pathological state" remains relatively constant when determined at intervals of several days (18). *Considerable variations of serum phosphatase may therefore confidently be ascribed to deviations from the normal physiological state, when observed in experiments in which no tissue or organ injury is produced, or to changes in pathological conditions observed experimentally or clinically.*<sup>6</sup>

In view of the valuable clinical data reported by Kay, it is unfortunate that his unit was based on a 48 hour incubation period. A different unit was proposed by Jenner and Kay, which they stated to be "about 1/50 of the old unit," this figure being derived from a ratio of *averages* obtained by the old and new methods in parallel analyses of normal adult plasma. This comparison of the two units is not borne out by the ratios of individual data in their ten determinations, which varied between 0.027 and 0.017

<sup>6</sup> Interpretation of clinical results may be facilitated by the following summary: normal adults, 1.5 to 4.0 units per 100 cc.; generalized osteoporosis, 5 to 10 units (1.5 to 4.0 in senile osteoporosis); clinical hyperparathyroidism, about 25 units; localized Paget's disease of bone, 5 to 20 units; polyostotic Paget's disease, 50 to 135 units; normal children, 5 to 12 units; active rickets, 30 to 165 units, decreasing rapidly on effective dosage; healed rickets, 6 to 14 units. Serum phosphatase is generally increased in jaundice. A detailed presentation and discussion of our clinical results will be published.

(average 0.02). Jenner and Kay evidently accept larger errors than need be tolerated. In three plasmas high in phosphatase (Paget's disease and osteitis fibrosa) the ratios were respectively 0.031, 0.029, and 0.035, indicating a very great inhibition of phosphatase activity.

Our unit is essentially Levene and Dillon's, but is based on incubation at 37° instead of 30°. We believe the former most desirable in serum phosphatase determinations. When shorter or longer incubation periods were employed instead of the standard 1 hour period, our conversion factors enabled us to calculate the analytical results in standard units. Our amplified definition of a unit is: Each unit of phosphatase activity is equivalent to the actual or calculated liberation of 1 mg. of phosphorus as the phosphate ion during the 1st hour of incubation at 37° and at pH 8.6, with a substrate containing sodium  $\beta$ -glycerophosphate, hydrolysis not exceeding 10 per cent of the substrate.

#### SUMMARY AND CONCLUSIONS

A convenient, simple, and rapid method for the determination of serum phosphatase has been developed. The relevant properties of phosphatase have been studied and errors due to various causes have been demonstrated and eliminated. The accuracy of the procedure justifies reliance upon it as a criterion of changes in the physiological state in experiments in which no tissue or organ injury is produced, or of changes in pathological conditions observed experimentally or clinically.

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## THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

### XXXII. ISOLATION OF TREHALOSE FROM THE TIMOTHY-GRASS BACILLUS\*

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#### INTRODUCTION

As recorded in Paper XXIII (1) of this series, the alcohol-ether extract of the timothy-grass bacillus, *Mycobacterium phlei*, contained in addition to phosphatide and fat a relatively large amount of polysaccharides. We are now engaged in an investigation of the polysaccharide fraction and have found that at least two, and probably three, different carbohydrates are present in the crude material. We have succeeded in isolating one of the component sugars in pure form and our results are briefly presented in this report.

The crude polysaccharide was acetylated with acetic anhydride in pyridine solution according to the procedure used by du Mont and Anderson (2) and by Ludewig and Anderson (3) in their investigations of the polysaccharides of the avian and human tubercle bacilli. The crude acetyl product, when dissolved in hot absolute methyl alcohol, deposited a generous crop of prismatic needle-shaped crystals after the solution had cooled. The crystalline acetyl derivative, after it had been purified by several recrystallizations, was saponified with dilute barium hydroxide and

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† Holder of a National Tuberculosis Association Fellowship at Yale University, 1932-33.

the free sugar was isolated. When the sugar was recrystallized from 80 per cent alcohol it was obtained in the form of magnificent, colorless, rhombic crystals. The sugar was found to be identical in crystal form, melting point, and in optical rotation with pure trehalose.

The more soluble portion of the acetyl derivative has not yet been isolated in pure form. Preliminary examination of the material indicates that it is a mixture of more complex polysaccharides which give the color reactions for pentose sugars.

Bacteria are classified with the group of plants known as fungi which are free from chlorophyll. The disaccharide trehalose is the characteristic carbohydrate of fungi. The occurrence of trehalose in the timothy-grass bacillus is therefore not surprising. In view of our present observation it would seem reasonable to expect that tubercle bacilli and other acid-fast organisms, as well as ordinary bacteria, will be found to contain trehalose as one of their characteristic carbohydrates. Investigations on this subject are now under way in this laboratory.

#### EXPERIMENTAL

*Preparation of Acetyl Derivative*—A portion of the crude polysaccharide, weighing 33 gm., was treated at room temperature with a mixture of 500 cc. of pyridine and 275 cc. of acetic anhydride. After 3 days the solid had dissolved completely, yielding a dark brown solution. The solution was poured into 2.5 liters of ice water, when a voluminous amorphous precipitate separated. After the mixture had stood for 2 hours, the precipitate was filtered off, washed with water, and dried in a vacuum desiccator.

The dried material which weighed 35 gm. was dissolved in hot absolute methyl alcohol and a trace of insoluble matter was filtered off. Colorless prismatic needles separated from the solution on cooling. The crystals were filtered off and washed with cold methyl alcohol. The filtrate was concentrated and cooled, when a second crop of crystals was obtained and combined with the first lot. The total yield of crude crystals was 14 gm., corresponding to 40 per cent of the acetylation product. The crystals were dissolved in hot methyl alcohol and the solution was treated with norit, filtered, and cooled. The crystals which separated were collected on a Buchner funnel and recrystallized three times

from ethyl alcohol. The colorless prismatic needles thus obtained weighed 11 gm. The substance melted not sharply at about 80°.

*Rotation*—0.1241 gm. of substance was dissolved in chloroform and made up to 10 cc. In a 1 dm. tube  $\alpha = +2.023^\circ$  at 22°.  $[\alpha]_D^{22} = +163^\circ$ .

The substance was again recrystallized two times from ethyl alcohol but there was no change either in the melting point or in the optical rotation.

*Analyses*<sup>1</sup>—Ash: 5.996 mg. of substance left 0.005 mg. of residue on combustion. The crystals are therefore practically ash-free. Molecular weight: 0.285 mg. of substance in 1.781 mg. of camphor gave a depression of 9.5°, corresponding to a molecular weight of 674.

0.1076 gm. substance: 0.0583 gm. H<sub>2</sub>O and 0.1938 gm. CO<sub>2</sub>

C<sub>28</sub>H<sub>38</sub>O<sub>18</sub> (678). Calculated. C 49.55, H 5.60

Found. " 49.12, " 6.06

*Acetyl Determination*—0.2026 gm. of substance was shaken in a tightly stoppered flask with 56.4 cc. of 0.2 N Ba(OH)<sub>2</sub> until everything was completely dissolved. The excess alkali was titrated with 0.2 N H<sub>2</sub>SO<sub>4</sub>. 11.86 cc. of 0.2 N Ba(OH)<sub>2</sub> had been required for the saponification, corresponding to 0.1019 gm. of CH<sub>3</sub>CO.

C<sub>12</sub>H<sub>14</sub>O<sub>11</sub> (CH<sub>3</sub>CO)<sub>8</sub> (678). Calculated. CH<sub>3</sub>CO 50.73

Found. " 50.29

*Isolation of Trehalose*—The pure octaacetate was saponified as described below. The recrystallized product, 10 gm., was dissolved in 600 cc. of alcohol and, after adding a slight excess of barium hydroxide dissolved in water, the solution was refluxed for 2 hours. After the reaction mixture had stood overnight most of the alcohol was distilled off under reduced pressure and the residue was diluted with water. The barium was then removed quantitatively with sulfuric acid and the barium sulfate was filtered off. The filtrate was concentrated under reduced pressure and finally dried to a thick syrup in a vacuum desiccator. The syrup was stirred up with absolute alcohol, when a white amorphous powder was obtained. The latter was filtered off, washed

<sup>1</sup> We are indebted to Professor H. T. Clarke of Columbia University for the microanalytical determinations.



with absolute alcohol, and dried. The substance, which weighed 4.9 gm., was dissolved in 20 cc. of water and 100 cc. of absolute alcohol were added. Crystallization was easily started by scratching, and after the solution had stood overnight in an ice box very large, colorless, rhombic crystals had separated. The crystals weighed 4.4 gm.

The powdered substance melted at 98–100°. On drying in a vacuum over phosphorus pentoxide at 61°, the loss in weight was 9.2 per cent, corresponding to 2 molecules of water of crystallization. The crystals were easily soluble in water. The solution was neutral to litmus and it gave no reduction when boiled with Fehling's solution.

A portion of the substance was refluxed with 5 per cent sulfuric acid for 2.5 hours. The solution was neutralized and the reducing sugar, determined by the Munson-Walker gravimetric method, corresponded to 96 per cent calculated as glucose.

*Rotation of Dihydrate*—0.1040 gm. of substance was dissolved in water and made up to 10 cc. In a 1 dm. tube  $\alpha = +1.899^\circ$  at 22°  $[\alpha]_D^{22} = +182.6^\circ$ . The calculated specific optical rotation of the anhydrous sugar is therefore  $+201^\circ$ .

#### *Analysis*

0.0936 gm. dried substance: 0.0569 gm. H<sub>2</sub>O and 0.1441 gm. CO<sub>2</sub>

C<sub>12</sub>H<sub>22</sub>O<sub>11</sub> (342). Calculated. C 42.09, H 6.43

Found. " 41.98, " 6.80

It is evident from the data presented that the properties of the crystalline sugar, obtained from the crude polysaccharide isolated from the alcohol-ether extract of the timothy-grass bacillus, correspond to those of trehalose. It may be noted, however, that a discrepancy exists in regard to the melting point of the octa-acetate which was found to be 80°, whereas trehalose octaacetate is stated to melt at 96°. Acetyl derivatives were therefore prepared from the sugar obtained from the timothy-grass bacillus and from commercial trehalose by refluxing with acetic anhydride and fused sodium acetate. Both products were treated exactly alike and crystallized from ethyl alcohol. The yield was 94 per cent. Both preparations melted not sharply at 82–85° and this melting point was not changed by several further crystallizations. The rotation of both preparations was  $+163^\circ$ . Drying the

preparations at 61° and later at 78° in a vacuum over phosphorus pentoxide caused no loss in weight but it was found that the melting point of both preparations was 98–99° after this treatment. A mixture of the two preparations also melted at 98–99°. Further heating at 78° for 14 hours caused no change in this higher melting point and it had no effect on the optical rotation, which was +163°.

#### SUMMARY

The alcohol-ether extract of living moist timothy-grass bacillus contains in addition to lipids a considerable amount of polysaccharides.

After acetylation of the crude polysaccharide it was possible to isolate about 40 per cent of the acetyl derivative in the form of the crystalline trehalose octaacetate.

Crystalline trehalose was prepared from the acetyl derivative.

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# STANDARDIZED METHODS FOR THE DETERMINATION OF URIC ACID IN UNLAKED BLOOD AND IN URINE

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## INTRODUCTION

This paper is the outcome of a comprehensive critical review of the colorimetric method for the determination of uric acid both in blood and in urine. The work began with the observation that we were no longer able to obtain such complete recoveries of uric acid added to unlaked blood as had been previously reported (1). The cause of our failure was soon found. The Folin-Marenzi method for the preparation of the uric acid reagent free from phenol reagent proved inadequate when applied to the Merck's "reagent sodium tungstate" now in the market. The reagents invariably gave some color with tyrosine and some color with the urea-cyanide and yielded therefore necessarily only a very narrow range of true proportionality between different amounts of uric acid. Apparent losses of as much as 15 per cent could thus be encountered, all due to the fact that the range of true proportionality fell short of what it should have been. It therefore became necessary first of all to improve the method for the preparation of a dependable uric acid reagent. The subsequent check work gradually developed into revisions of the several uric acid methods sponsored by this department.

### *Revised Process for Preparation of Uric Acid Reagent Free from Phenol Reagent*

In the Folin-Marenzi (2) process for the preparation of this reagent the larger part of the molybdenum sulfides is precipitated and removed by filtration and the soluble sulfides are removed from the filtrate by extraction with alcohol. In the process de-

scribed below only a minute fraction of the sulfides is rendered insoluble.

The first step in the preparation should be to dissolve a few crystals of the tungstate and test the reaction with phenolphthalein solution to make sure that it is permanently alkaline to phenolphthalein. If it is not alkaline, it is apt to be of poor quality in other ways, especially with respect to its molybdenum content, and at all events, it should first be rendered alkaline by boiling with a slight excess of sodium hydroxide.

Transfer 100 gm. of sodium tungstate (of the requisite alkaline reaction) and 150 cc. of water to a 500 cc. Florence flask. Dilute 20 cc. of phosphoric acid with 50 cc. of water and pour this gradually and with shaking into the tungstate-water mixture. Shake until the tungstate has dissolved and cool under running water. Pass  $H_2S$  into the solution for 10 minutes.

Transfer the solution to a 500 cc. separatory funnel and add (gradually at first) with gentle shaking a total of 150 cc. of alcohol. Shake vigorously for 7 to 8 minutes. Let the mixture settle and then withdraw the more or less yellow bottom layer, the weight of which should be 160 to 170 gm.

Discard the highly colored upper layer and rinse the separatory funnel. Return the phosphotungstate solution, together with 100 cc. of rinsing water, to the separatory funnel. Add 75 cc. of alcohol and shake thoroughly as before. Withdraw the bottom layer, which should now be substantially colorless, into a weighed 500 cc. Florence flask and dilute the contents to a weight of about 250 gm. Boil vigorously for 5 minutes to remove the  $H_2S$ . Dilute again to a weight of 250 gm. and add 15 cc. of phosphoric acid (85 per cent). Boil under a reflux condenser for 1 hour. Remove the condenser, add a little liquid bromine or strong bromine water, and boil another 5 minutes to remove the surplus bromine. Cool and dilute to a volume of 500 cc.

The directions given above for the preparation of the uric acid reagent are the outcome of a prolonged study and a few explanatory remarks may prove helpful.

1. The total volume of 85 per cent phosphoric acid used with 100 gm. of sodium tungstate is only 35 cc., instead of 50 cc. The reasons for this change are as follows: No initial mixture of sodium tungstate and phosphoric acid could be found in which treatment

with  $H_2S$  converts *all* of the molybdenum present into sulfides which are either insoluble or completely extractable with alcohol. There always remain some molybdenum compounds which may be converted into the phenol reagent during the final boiling period. The greater the acidity or excess of phosphoric acid in that boiling mixture the more extensively are the molybdenum compounds present converted into phenol reagent. The uric acid reagent, however, begins to form in the presence of a sufficient excess of phosphoric acid to give a positive reaction with Congo red paper (30 cc. of 85 per cent phosphoric acid to 100 gm. of sodium tungstate) and a very small excess of phosphoric acid above that amount is sufficient to yield a practically quantitative conversion into the uric acid reagent, at the dilution indicated in the directions. With the slight excess of free phosphoric acid present when 35 cc. are used, no phenol reagent is formed when only traces of molybdates are present as is the case after the treatment with  $H_2S$ . In the presence of larger amounts of molybdates, however, even this slight excess of acidity will produce disastrous amounts of phenol reagent. With Merck's "reagent sodium tungstate" as it was a few years ago, for example, a nearly perfect uric acid reagent can be obtained by boiling 100 gm. with 33 to 35 cc. of phosphoric acid, but the corresponding product now in the market is unusually rich in molybdate. It is labeled, "according to Dr. Folin." The older good brand carries no such description.

2. The highly concentrated solution of the uric acid reagent remaining in the boiling flask after the surplus bromine has boiled off has a lemon-yellow color, but as the solution is cooled most of the color fades away, so that after diluting to 500 cc., the solution has only a just perceptible yellow tint—if the solution is free from phenol reagent. The phosphomolybdates on the other hand are intensely yellow, especially so in the case of the phenol reagent, the phospho-18-molybdic acid. Any solution of the uric acid reagent which has a distinctly yellow color is, therefore, surely contaminated with phenol reagent, and it will invariably be found to yield a blank (blue color) when 4 cc. are added to a mixture of 5 cc. of water and 10 cc. of urea-cyanide solution.

Concentrated solutions of the uric acid reagent completely free from phosphomolybdates are probably quite colorless and the faint yellowish tint left in the uric acid reagents prepared according

to the directions given above are almost certainly due to traces of phospho-24-molybdic acid which, under the given conditions, are prevented from transformation into the phenol reagent.

3. This uric acid reagent contains no added lithium salt to prevent the formation of turbidities.

*Urea-Cyanide Solution*—The urea-cyanide solution, described in 1930, can be used without any change in connection with the revised method for the direct determination of uric acid in unlaked blood filtrates, but it does not always yield 100 per cent of the uric acid precipitated by means of silver lactate. Accordingly, it has been modified so as to meet these additional requirements.

The urea-cyanide solution which we now use is prepared as follows:

Transfer 75 gm. of Merck's Blue Label sodium cyanide to a 2 liter beaker, add 700 cc. of water, and stir until the cyanide is completely dissolved. Add 300 gm. of urea and stir. Then add 4 to 5 gm. of calcium oxide and stir for about 10 minutes. Filter, at once if necessary for immediate use, but preferably not until the next day. Add to the filtrate about 2 gm. of powdered lithium oxalate, shake occasionally for 10 to 15 minutes, and filter.

Lithium oxalate is better than the disodium phosphate which we formerly used for the removal of the dissolved calcium hydroxide. Its solubility in the urea-cyanide solution is slight, yet great enough to transform the dissolved calcium hydroxide into the insoluble oxalate. Sodium oxalate powder cannot be used, because it lacks the required solubility, and potassium oxalate is, of course, unsuitable.

The lithium oxalate is prepared as follows: Transfer 50 gm. of lithium carbonate and 85 gm. of oxalic acid to a 3 liter beaker. Pour on the mixture about 1 liter of hot water (70°). Stir cautiously to avoid loss by foaming until the evolution of CO<sub>2</sub> ceases. Add 1 liter of alcohol and filter on a Buchner funnel.

#### *Revised Macromethod for Determination of Uric Acid in Blood*

Since it had been seen in the early part of this work what serious errors can creep into colorimetric analyses merely because the range of true proportionality in the color reaction is too narrow, the attempt was made once more to secure a wider range of true proportionality than has yet been obtained in colorimetric deter-

minations of uric acid in blood, and these endeavors have resulted in a modification of the method which most workers will probably accept as an improvement.

The obvious way to try to increase the range of true proportionality, if one has a uric acid reagent and cyanide which give no blanks, was to use larger quantities of the uric acid reagent and cyanide for the development of the color. There is a limit, however, to the amount of uric acid reagent to be used, even with the efficient urea-cyanide solution, without getting turbidities when the mixtures are heated. But if the heating is omitted, any desired quantity of the uric acid reagent may be used together with the urea-cyanide solution without getting turbidities. By the simple expedient of using more of the reagents and omitting the heating, the range of true proportionality has been greatly increased. Colorimetric readings between 35 and 10 mm., when the regular standard is set at 20 mm., have now become dependable, although it is better to repeat the determination with less than 5 cc. of blood filtrate when the first determination yields colorimetric readings of 10 mm. or less as indicated on p. 119. While the determination now takes a little more time, because of the longer waiting period, it really takes less work and less attention.

We now make the regular (macro-) determination of uric acid in blood in the following manner: transfer 5 cc. of unlaked blood filtrate to a test-tube graduated at 25 cc. and transfer to another similar test-tube 5 cc. of the standard uric acid solution containing 0.02 mg. of uric acid. With a cylinder, add 10 cc. of the urea-cyanide solution. Mix by whirling the test-tubes at an angle of about 60°. Add 4 cc. of uric acid reagent of double the regular concentration. Let stand for about 20 minutes. Dilute to volume, mix, and make the color comparison.

$(20/x) \times 4$  gives the uric acid content of the blood in mg. per cent when the standard is set at 20 mm.  $x$  represents the colorimetric reading of the blood filtrate.

The uric acid reagent should be added at the same time to the standard and the unknown and the tubes should be in a vertical position when the reagent is added so that it does not flow down one side.

The maximum obtainable color is not quite reached during a 20 minute waiting period, and if it is more convenient, one can just



as well wait for 40 minutes or longer before finishing the determination, but the colorimetric readings obtained at the end of 20 minutes are reliable.

*Revised Micromethod for Determination of Uric Acid in Blood*

While checking up the macromethod, parallel determinations were also made with the micromethod described by Folin and Svedberg (3) and, as was to be expected, the variations and errors in recovery experiments were distinctly larger than with the macromethod. In the micromethod one is working with less than one-half as much uric acid as in the macromethod, and the errors due to the color obtained from the reagents alone necessarily become more significant. These difficulties disappeared after we had succeeded in obtaining uric acid reagents which gave no color with the urea-cyanide solution and water.

The modified micromethod corresponding to the macromethod for the determination of uric acid in blood is as follows:

The blood filtrate for the determination of the uric acid by the micromethod is obtained by adding 0.2 cc. of blood to 4 cc. of tungstate-sulfate mixture in a centrifuge tube and 15 minutes later adding 1 cc. of sulfuric acid and centrifuging.

The sulfate-tungstate solution contains 20 gm. of anhydrous sodium sulfate and 3 gm. of sodium tungstate per liter. The sulfuric acid solution is obtained by diluting 12 cc. of  $\frac{2}{3}$  N sulfuric acid to 100 cc.

Transfer 4 cc. of the extract to a test-tube graduated at 25 cc. To two other similar test-tubes add 4 cc. and 2 cc. (plus 2 cc. of water) of a standard uric acid solution containing 1 mg. of uric acid in 500 cc.

To each of the three tubes add 10 cc. of the same urea-cyanide solution as is used in the macromethod and mix. Then add 4 cc. of the concentrated uric acid reagent and let stand for 20 to 30 minutes. Dilute to volume, mix, and make the color comparison.

$(20/x) \times 5.2$  (or 2.6) gives the uric acid in mg. per 100 cc. of blood.

It will be noted that in this calculation the filtrate is regarded as representing a dilution of 1 in 26 instead of 1 in 25 as given in the original method.

*Determination of Uric Acid in Blood by Indirect Method*

When uric acid is determined colorimetrically directly on blood filtrates the process used is called the direct method. The process involving a preliminary precipitation of the uric acid has therefore become the "indirect method." The direct method was first introduced by S. R. Benedict, in 1922, and was promptly adopted by Folin, but with the reservation that the indirect method should be retained at least for check purposes. The indirect method is still described in Folin's "Laboratory manual," but as a matter of history it fell at once into complete disuse and all subsequent efforts have aimed at improving the direct method—and this notwithstanding the fact that uric acid added to blood has never been quantitatively recovered by the direct method when applied to the filtrates from laked blood. By the application of a uric acid reagent free from phenol reagent to the filtrates from unlaked blood the errors and uncertainties of the direct method were finally completely removed (if the recovery of added uric acid can be accepted as an adequate criterion of trustworthiness). But this time it was decided, nevertheless, to go back once more to the preliminary precipitation of the uric acid with acid silver solution.

The completeness of the precipitation of uric acid by silver lactate in the presence of a little chloride even from the most dilute blood filtrates has never been questioned. It is probably the only strictly quantitative known precipitant for uric acid. One exception could be taken to these statements. In 1922, it was admitted that only 90 to 95 per cent of the dissolved urates could be recovered from very dilute solutions in water (4). At that time it was not so clearly understood as now that such losses as did occur might be due to some factor interfering with the color reaction rather than to incomplete precipitation. We have now reexamined that problem and have found that the apparent losses are due to the depressing effects of the dissolved silver on the color reaction. By cutting down the chloride or by using a more effective cyanide solution or by extracting the silver precipitate with acid chloride the losses vanished.

In applying the indirect method to unlaked blood filtrates we have used partly the whole silver precipitate dissolved in the urea-cyanide solution and partly only the acid chloride extract from the precipitate.

5 cc. of the blood filtrate in a 15 cc. centrifuge tube were precipitated with 2 cc. of the acid silver solution described below (p. 121) and centrifuged at once. The precipitates were then either extracted with 1 cc. of a 10 per cent solution of sodium chloride in 0.1 N hydrochloric acid and washed with 4 cc. of water or were dissolved in 10 cc. of urea-cyanide solution and the tubes rinsed with 5 cc. of water. In other respects the determinations were made as described above for the direct macromethod. The ex-

TABLE I

*Showing Recovery of Added Uric Acid and Also Agreement between Results Obtained by Direct Method, Indirect Method, and Micromethod on Filtrates from Unlaked Human Blood*

The figures indicate the uric acid found before and after adding 5 mg

Sample No	A	A + 5	B	B + 5	C	C + 5	D	D + 5
1	3 5	8 3	3 44	8 2	3 5	8 2	3 49	8 44
2	3 2	8 15	3 1	7 85	3 2	8 1	3 1	8 0
3	3 7	8 4	3 55	8 15	3 6	8 3	3 6	8 4
4	2 05	6 95	2 0	7 0	2 1	6 8	2 1	7 0
5*	3 2	8 2	3 2	7 65	3 2	7 65	3 3	8 0
6	3 5	8 36	3 5	8 2	3 5	8 1	3 5	8 4
7*	2 9	7 65	2 9	7 44	2 9	7 65	3 0	7 85
8	2 9	7 8	2 9	7 75	2 9	7 75	3 1	8 05
9	2 05	7 0	2 1	6 9	2 0	7 04	2 05	7 0
10	2 3	7 15	2 3	7 0	2 3	7 15	2 35	7 2

A = direct macromethod; B = determination on the silver precipitate; C = determination on the extract from the silver precipitate; D = micromethod.

\* Not repeated with less than 5 cc. of filtrate

traction method calls for two extra centrifugings and requires neat work, but if carefully done gives all the uric acid.

All through the rather long period covered by this research, Dr. C. L. Derick, of the Peter Bent Brigham Hospital, has supplied us with all the hospital bloods we could use. In view of the very great demand for space in this *Journal* the original plan of giving a comprehensive series of analyses has been abandoned. In Table I we give therefore in highly condensed form, only a few analyses of bloods in which the uric acid was determined before and after the addition of uric acid, by each of the four methods described

above. The significance of these figures is so clear that comments would seem to be superfluous. It need be stated, however, that the first macrodeterminations made on the filtrates containing added uric acid would sometimes be too low by 0.2 to 0.3 mg. per cent, but these deficits disappeared when the determinations were repeated with less than 5 cc. of the blood filtrates. In other words, when 8 mg. or more of uric acid are found by the regular method involving the use of 5 cc. of blood filtrate and the 0.02 mg. standard, the determination should be repeated with less than 5 cc. of blood filtrate.

For the sake of convenience and greater accuracy the added uric acid was added to the tungstate-sulfate solution used for the precipitation of the protein and not directly to the blood, but previous experience has shown that this makes no difference as to the recovery.

#### *Determination of Uric Acid in Urine*

For clinical purposes the determination of uric acid in urine has been almost entirely replaced by determinations of the uric acid in blood and for this reason there has been very little recent investigation or revision of the current methods as applied to urine. What little check work has been done has consisted of parallel determinations on urine by the three currently accepted methods, and since all of these have yielded substantially identical values, the choice of working method adopted and recommended in different laboratories has been based on minor considerations of convenience or other supposed advantages. The fundamental question whether the indirect methods involving a preliminary precipitation do quantitatively recover uric acid from known solutions has seemingly received very little attention. For the quantitative (100 per cent) recovery of minute amounts of uric acid (0.02 mg.) from 5 to 7 cc. of solution the slightly acid silver reagents are probably the only ones which are strictly dependable. No figures seem to be available showing the minimum amount of uric acid which can be quantitatively recovered by other precipitants.

The acid silver precipitation has been criticized from time to time. Because the uric acid precipitation is always accompanied by the precipitation of silver chloride it has been thought that the uric acid is merely carried down by some adsorption process and

does not depend on the insolubility of silver urate in slightly acid solutions. Silver urate is, however, less soluble in slightly acid than in strongly ammoniacal solutions, though this advantage may be lost if too strongly acid silver solutions are used. With acid silver solutions and 0.5 mg. of uric acid one obtains the same sort of gelatinous silver urate in the absence of a chloride as is obtained with ammoniacal silver solutions. The silver chloride serves two useful purposes. It protects the silver urate from the oxidizing effects of the surplus silver salt and it makes it possible to isolate by means of the centrifuge the silver urate in such minute quantities that they could not possibly be so isolated in the absence of the silver chloride. The absence of the latter condition sets a limit to the minimum amount of uric acid which can be isolated by means of the ammoniacal silver precipitation.

The amount of silver chloride precipitated together with the uric acid must not be too large. It should not exceed that derived from 10 mg. of NaCl and, preferably, it should be somewhat less, because much silver tends to depress the color obtained from the uric acid.

The zinc hydroxide precipitation resembles the acid silver precipitation in that it also yields a urate enclosed in another precipitate which can be isolated by help of the centrifuge. But the zinc urate is apparently somewhat more soluble than the silver urate and hence does not yield 100 per cent recovery with as little as 0.02 mg. of uric acid (from 5 cc. of the standard uric acid solution).

The fact that the precipitation of silver urate and zinc urate is due to the insolubility of these salts rather than to adsorption phenomena does not exclude the possibility that these precipitates may contain materials, other than uric acid, capable of reacting with the uric acid reagent. In the course of this work we have in fact satisfied ourselves that the silver precipitates as heretofore obtained from practically undiluted urine must have contained some other reacting material besides uric acid (see p. 123). We have not investigated the zinc precipitate or the ammoniacal silver precipitate from this point of view.

Another series of criticisms of the acid silver precipitation of uric acid is based on the darkening of the silver precipitates. It is certainly true that this darkening, if pronounced, is apt to involve destruction and loss of uric acid. With a modicum of care this

darkening is not encountered when working with blood filtrates or diluted urine, but when working with 1 cc. of urine the silver precipitates are often dark gray in color, even when no undue lapse of time or exposure to light intervenes between the precipitation and the solution of the precipitate in the cyanide solution. Some urines contain reducing materials which have a very rapid reducing effect on the surplus silver lactate and if delay or more than a minimum exposure to light occurs, the precipitates will be black; in such cases loss of uric acid is an inevitable result, as has been pointed out recently by Christman and his coworkers (5). If the centrifuging is made immediately (as it always should be since the uric acid precipitation is instantaneous) some black sediment may form during the centrifuging process and it will be deposited partly on top of the nearly white silver precipitate. The black sediment so formed has little if any effect on the uric acid.

These facts simply constitute an unavoidable limitation on the acid silver precipitation of uric acid in urine. They mean that the centrifuging must be begun immediately after the addition of the silver and that no undue delay may intervene from the time the silver has been added until the precipitate has been completely dissolved in the cyanide solution. Directions of this sort are just as legitimate as are the directions to wait a certain length of time for the development of the color reaction.

The degree of acidity at which the silver precipitate is produced affects to a considerable extent the speed with which the surplus silver is reduced, but there is a limit to the permissible acidity if one does not want to encroach in the slightest degree on the insolubility of silver urate.

Our uric acid precipitations on blood filtrates were made mostly with a reagent, 1 liter of which contained 25 gm. of silver nitrate and 5 cc. of lactic acid which had been partly neutralized by boiling with 5 gm. of  $\text{Na}_2\text{CO}_3$ . After a few days exposure to sunlight and filtering, this reagent keeps fairly well. When used only for occasional determinations, it would not be safe to use this reagent without filtering, and many efforts were made to find a suitable silver solution of unlimited keeping quality, but no solution could be found which was entirely satisfactory. The conditions were altered, therefore, so as to permit the use of silver nitrate without the addition of any organic acid.

One objective in this study was to adapt the method to the needs of hospital laboratories where they now make at least twenty-five uric acid determinations on blood for every such determination made on urine. To this end we have sought to make the method as nearly identical as possible with the method used for blood, including the use of the same utensils and reagents, except for one separate step, namely the preliminary precipitation of the uric acid.

Of course, we have not been unmindful of the possibility that the preliminary precipitation of the uric acid might be omitted, thus making its determination in urine completely similar to the determination in blood filtrates. Past efforts in this direction, made in our laboratory, have always yielded unacceptable results, but it seemed possible that by working with no more than 0.015 to 0.04 mg. of uric acid, the various disturbing factors might be reduced to the vanishing point. These most favorable conditions have not been used in any previously published direct method for the determination of uric acid in urine. From theoretical considerations both the positive and the negative sources of error, recently elucidated by Christman and his coworkers (5), might become negligible under the new conditions for the determination of minute amounts of uric acid.

The indirect method, described below, should yield results, the accuracy of which should be beyond question, and we hoped to use it as a standard by which to judge the validity of the direct method. Parallel determinations were made also by a suitable modification of Folin's method based on the use of 1 (or 2) cc. of undiluted urine.

Conflicting results were obtained. The direct method and Folin's macromethod usually gave identical values with normal urines, but these values were nearly always from 5 to over 10 per cent higher than those obtained by the indirect method applied to the diluted urines.

In order to settle the difficulty represented by these discrepancies, we have adopted a procedure by which the validity of any method for the determination of uric acid in human urine may be controlled, without having recourse to the unconvincing process of merely checking one method by another which may or may not be more dependable.

Transfer 0.3 gm. of oxalic acid and 3 gm. of Lloyd's reagent to a small flask, add 25 to 30 cc. of urine, shake immediately for about 2 minutes, and filter. All of the uric acid (up to 25 mg.), nearly all of the creatinine, and very little else, are removed by this treatment.

(a) If 2 cc. of this uric acid-free urine be diluted to 100 cc. with water, 5 cc. will yield considerable color with 10 cc. of the cyanide solution and 4 cc. of the uric acid reagent, whereas the silver precipitate from 5 cc. (plus 0.5 cc. of acid chloride solution) will yield no color.

(b) If 2 cc. of the undiluted uric acid-free urine be added to 5 cc. of water in a centrifuge tube and precipitated with acid silver solution or silver nitrate, the precipitate will yield a color with cyanide-carbonate-urea solution and uric acid reagent.

(c) If suitable amounts of standard uric acid solution are added together with the water in these tests with uric acid-free urine, a correct and dependable method must yield 100 per cent recovery (and no more), just as it would, or would be expected to do, if no uric acid-free urine were present. The validity of these tests is increased, if one works, as we have done, with twice as much uric acid-free urine as the volume of untreated urine actually used in a determination.

By the application of these tests to the three methods included in this investigation, it was found that the indirect method described below, and that method only, yields 100 per cent recovery (and no more) of uric acid added to uric acid-free urine. We are therefore inclined to consider this as a standard method, the first really standardized method for the determination of uric acid in urine. Other methods in current use could of course be subjected to the same test, but it has seemed better to leave that work to those who are more interested in the other methods. In such tests, the amount of uric acid to be added and recovered is of course different for different methods.

In these experiments with uric acid-free urines, as in actual uric acid determinations, the direct method, described below, has yielded recoveries and values of about the same order of accuracy as the corresponding figures by Folin's older method based on undiluted urine. But, as already indicated, these figures are apt to be too high by from 5 to 10 per cent and occasionally even more.



And these errors are likely to be larger still, if the uric acid reagent is not completely free from phenol reagent.

*Indirect Method*—Two solutions, in addition to those used in blood analysis, are required; both keep indefinitely.

1. A 5 per cent solution of silver nitrate. This solution, even if perfectly clear when first prepared, may develop a slight color on standing. This color is most quickly produced by heating to  $100^{\circ}$  for 2 hours in a flask covered with a beaker. After cooling, add a few cc. of a solution containing 50 mg. of sodium chloride, shake thoroughly, and filter through a double layer of quantitative filter paper until crystal-clear. Thereafter the solution will remain perfectly colorless and need not be kept in brown bottles.

2. A solution containing 1 per cent of sodium chloride, 2 per cent of crystallized sodium acetate, and 1 volume per cent of concentrated acetic acid (99 per cent).

Half fill a 100 cc. volumetric flask with water. With a Folin-Ostwald pipette introduce 1 cc. of the urine. Add 10 cc. of the chloride acetate solution and then, without shaking, so as to avoid foaming, dilute to the mark with water and mix.

From this diluted urine transfer 5 cc. and 3 cc., plus 2 cc. of water, to 15 cc. centrifuge tubes. Add to each 3 cc. of the silver nitrate solution and centrifuge at once fairly rapidly, for 4 to 5 minutes, so as to get perfectly clear supernatant solutions. A few tiny flakes may float on the surface, but these contain no uric acid. Decant and drain over a sink. It is permissible to let cold tap water rinse the mouth of the tube during the draining. Add to each tube 10 cc. of the urea-cyanide solution described on p. 114. Stir immediately (and simultaneously) with glass rods until the two sediments have completely dissolved. Transfer the silver cyanide solutions to test-tubes graduated at 25 cc., and rinse with exactly 5 cc. of water. Mix by whirling at an angle of about  $60^{\circ}$  until the solutions are visibly uniform. In another graduated test-tube place 5 cc. of the standard uric acid solution containing 0.02 mg. of uric acid together with 10 cc. of the urea-cyanide solutions and mix.

Add to each of the three tubes 4 cc. of the uric acid reagent described on p. 111 and let stand for 15 to 25 minutes. Dilute to volume, mix, and make the color comparison between the standard and the unknown which is nearest to it in depth of color. When

the standard is set at 20 mm., colorimetric readings between 35 and 10 mm. are acceptable.

*Direct Method*—Half fill a 100 cc. volumetric flask with water. With a Folin-Ostwald pipette introduce 1 cc. of urine, dilute to volume, and mix. Introduce into test-tubes graduated at 25 cc. 5 cc. of the diluted urine and 3 cc. of diluted urine plus 2 cc. of water. To another graduated test-tube add 5 cc. of the standard uric acid solution. Add 10 cc. of the urea-cyanide solution to each, mix, and add 4 cc. of the uric acid reagent. Then finish the determination as in the indirect method.

The merely diluted urine used in the direct method can be used also for the indirect method, by adding 0.5 cc. of the chloride-acetate solution, before precipitating with the silver nitrate.

In conclusion, it must be stated that dependable uric acid values cannot be obtained from urines which contain much bile. Neither the indirect method described in this paper nor any other known method will yield exactly 100 per cent recovery of uric acid added to such urines after the preliminary removal of the preformed uric acid. After a number of unsuccessful attempts were made to produce a special method for use with such urines, the project was abandoned, at least for the present. Taken by itself, the problem represented by icteric urines is probably of slight importance, but it makes one wonder whether there may be other urines, the uric acid content of which cannot be determined with precision. Urines of some herbivorous animals may well be a case in point. The identification of such urines can probably always be made by means of recovery experiments such as have been outlined in this paper.

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## THE PREPARATION OF ALIPHATIC CHOLESTERYL ETHERS AND CHOLESTERILENE

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The preparation of aliphatic cholesteryl ethers has not been successful with the method of double decomposition of cholesteryl chloride and sodium alcoholate, or sodium cholesterolate and halogen alkyl (1, 2). During such reactions hydrochloric acid is split off with the production of cholesterolene, or resinous products.

The method of Obermüller (3), whereby the benzylcholesteryl ether is obtained through the action of benzyl chloride on sodium cholesterolate, fails when the simpler halogen alkyls are employed (Diels and Blumberg (4), Steinkopf and Blümner (2)). Diels and Blumberg, however, solved the problem by heating magnesium, the alcohol, and cholesteryl chloride in a bomb. Under these conditions little hydrochloric acid appears. Very recently Wagner-Jauregg and Werner (5) have been able to prepare the methyl and ethyl ethers by long heating of cholesteryl chloride with alcohol in a bomb.

Methyl ethers of ergosterol may be prepared by the interaction of sodium ergosterolate with methyl iodide, as Heilbron and Simpson have recently shown (6).

Bills and McDonald (7) have described a method for the preparation of ethers with the higher alcohols, isoamyl to secondary octyl, in which floridin is used as catalyst. Cholesterol is dissolved in benzene, toluene, or xylene and heated with the alcohol and floridin.

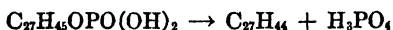
Very recently Stoll (8) has prepared ethers by heating the *p*-toluene sulfonic acid ester of cholesterol for several hours with the alcohol.

We have been able to prepare ethers with good yields by heat-

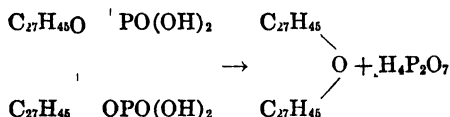
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ing monocholesterylphosphoric acid with the alcohol and sulfuric acid. Heating an alcoholic solution of cholesterol with sulfuric acid and phosphoric acid, when methanol or ethanol was used, did not lead to satisfactory results. However, by employing propyl alcohol a small amount of ether could be obtained. The heating of a solution of cholesterol in isopropyl alcohol plus 10 per cent of concentrated sulfuric acid gave a fair yield of this ether.

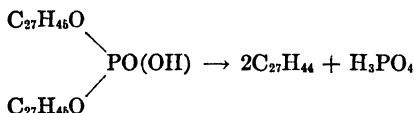
*Preparation of Cholesterilene*—If monocholesterylphosphoric acid is heated to melting, decomposition takes place with the liberation of phosphoric acid and production of cholesterilene. We suppose the reaction to be as follows:



The reaction is not simple, as dicholesteryl ether was also found among the products.



We have also obtained cholesterilene from dicholesterylphosphoric acid, but in this reaction the formation of the dicholesteryl ether was not observed.



The yield of cholesterilene is from 50 to 60 per cent of the theoretical and the product requires very little further purification.

The absorption curves of cholesterilene prepared from dicholesterylphosphoric acid were measured by Dr. Wilhelm Menschick in this laboratory. In the short wave region they were found to correspond with the curve of cholesterilene published by Heilbron, Morton, and Sexton (9) (prepared by the method of Mauthner and Suida). The high maximum at  $240\mu\mu$  was always found but in place of the three maxima in the long wave region we have found increased transparency.

## EXPERIMENTAL

Moncholesterylphosphoric acid was prepared according to the method of von Euler, Wolf, and Hellström (10). Our preparation melted at 193–193.5°;  $\alpha_D^{20}$  (chloroform) =  $-35.64^\circ$ . A combination of the methods of von Euler and Bernton (11) and von Euler, Wolf, and Hellström was employed for the preparation of dicholesterylphosphoric acid. An equivalent amount of phosphorus oxychloride was added to a solution of cholesterol in pyridine; acetone was then added, the product filtered off, and heated with water. Recrystallization from hexane-methanol gave a substance with a melting point of 203–203.5° and  $\alpha_D^{23}$  (chloroform) =  $-34.5^\circ$ .

*Methyl Cholesteryl Ether*—10 gm. of moncholesterylphosphoric acid and 150 cc. of methyl alcohol (containing 10 volumes per cent of concentrated sulfuric acid) were heated at boiling for 12 hours. After about 2 hours the clear solutions became cloudy, fine oily droplets settled out, and on cooling a crystal cake formed on the bottom of the flask. The white crystals were washed with methanol, dried on porous porcelain, and recrystallized from ether-alcohol. 6 gm. of rod-like crystals were obtained.

$C_{28}H_{48}O$ . Calculated. C 83.92, H 12.08  
Found. " 83.91, " 12.33

M. p. = 84.5–85°;  $\alpha_D^{20}$  =  $-45.82^\circ$  (chloroform)  
Iodine No. = 63.66 (theoretical, 63.4)

All iodine numbers were determined by the Rosenmund-Kuhn-henn method (12).

*Ethyl Cholesteryl Ether*—This was prepared by the same method as described for methyl cholesteryl ether except that ethyl alcohol was used.

$C_{29}H_{50}O$ . Calculated. C 83.98, H 12.16  
Found. " 83.71, " 12.27

M. p. = 88.5°;  $\alpha_D^{22}$  =  $-39.37^\circ$   
Iodine No. = 59.34 (theoretical, 61.3)

*n-Propyl Cholesteryl Ether*—1 gm. of moncholesterylphosphoric acid was heated with 20 cc. of propyl alcohol (containing 5 cc. of concentrated sulfuric acid) for 5 hours. On cooling in

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ice-salt mixture a precipitate forms. After recrystallization from ether-ethanol there was a yield of 0.45 gm. of white needles.

$C_{30}H_{52}O$ . Calculated. C 84.03, H 12.23  
Found. " 83.88, " 12.40

M. p. = 100–100.5°;  $\alpha_D^{25} = -34.78^\circ$

Iodine No. = 60.85 (theoretical, 59.3)

When monocholesterylphosphoric acid is saponified in propyl alcohol containing 10 volumes per cent of sulfuric acid, after 2 hours heating the clear solution becomes cloudy and a white silky substance separates. There is no increase in this precipitate after about 8 hours of heating. It begins gradually to turn yellow and lose its crystalline character. The material was dried on porous porcelain and recrystallization attempted from chloroform and alcohol-ether. The product is slightly yellow-colored, practically insoluble in alcohol or ether, and melts at about 320°.

It seems probable that this substance is formed from the propyl ether, for if the reaction is stopped when the solution begins to cloud, the propyl ether can be isolated. The longer the heating is continued after the appearance of the turbidity, the smaller is the amount of ether that can be recovered. It is possible that the substance obtained on long heating is a dimeric cholesterilene. The same compound has also been obtained by heating cholesterol in propyl alcohol containing 10 volumes per cent of concentrated sulfuric acid.

$C_{27}H_{44}$ . Calculated. C 87.96, H 12.04  
Found. " 87.40, " 12.12

*Molecular Weight Determination (Rast)*—4.59 mg. substance: 68.00 mg. camphor (m.p. 175°); depression 3.5°; mol. wt. = 771.4

24.5 mg. substance: 312.0 mg. camphor; depression 4°; mol. wt. = 785, calculated for  $(C_{27}H_{44})_2 = 736.7$

*Isopropyl Cholesteryl Ether*—A solution of 3 gm. of cholesterol was heated 9 hours with 60 cc. of isopropyl alcohol containing 10 volumes per cent of concentrated sulfuric acid. Within 5 hours, turbidity appeared and after 7 hours large amounts of an oily product separated. Standing overnight in the ice chest caused the precipitation of a large crop of fine needles. After filtration, the needles were dissolved in ether, the ether solution washed with water, dried, concentrated, and alcohol added until turbidity

appeared. The solution was allowed to stand in the ice chest until crystallization occurred, the crystals were filtered off, and the filtrate was treated with a large amount of alcohol, whereby a second crop of crystals was obtained. Concentration of the mother liquor gave still more of the product. The combined crystal crops were recrystallized from alcohol-ether.

$C_{20}H_{32}O$ . Calculated. C 84.03, H 12.23

Found. " 84.09, " 12.40

M. p. = 132–132.5°;  $\alpha_D^{21} = -34.45^\circ$  (chloroform)

Iodine No. = 62.01 (theoretical, 59.3)

*Preparation of Cholesterilene from Monocholesterylphosphoric Acid*—Cholesterilene is best prepared from monocholesterylphosphoric acid by heating about 0.5 gm. in a stoppered test-tube by immersing in a sulfuric acid bath heated to 200°. Within a few seconds the substance melts and produces a light brown fluid. The tube is immediately removed from the bath, cooled, and the contents extracted with ether. The ether solution is washed with water, dried with calcium chloride, and the ether removed by distillation. The residue from the distillation is a brownish oil which is extracted with warm alcohol. The alcoholic extract deposits large transparent spear-shaped crystals on standing in the ice chest; these increase in number after 1 to 2 days. The yield was about 40 per cent. The mother liquor yielded a small extra crop of crystals which were impure.

M. p. = 76–78°;  $\alpha_D^{20} = -68.99^\circ$

Iodine No. = 73.36 (theoretical for 2 double bonds = 138.8; because the bonds are conjugated the actual  $I_2$  number is one-half the calculated (13)).

The residue from the alcohol extraction hardens to a yellow resinous mass after standing a day. This was dissolved in benzene and precipitated with ethanol. An amorphous powder was obtained with a melting point of about 190°. This product was not further investigated but it appears possible that it is the dicholesteryl ether obtained by Mauthner and Suida (14) during the preparation of cholesterilene by their method.

*Preparation of Cholesterilene from Dicholesterylphosphoric Acid*—The same method was used as described above. After distillation



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of the ether, the residual reddish brown oil was found to be completely soluble in alcohol. The yield was about 50 per cent.

$C_{27}H_{44}$	Calculated	C 87.96, H 12.04
	Found	" 87.74, " 12.18

M p =  $78.2^{\circ}$ ,  $\alpha_D^{18} = -77.53^{\circ}$

Iodine No = 77.87

### SUMMARY

1. A new method is described for the preparation of aliphatic cholesteryl ethers from monocholesterylphosphoric acid

2. Cholesterilene has been prepared with good yields by a new procedure in which phosphoric acid is split from mono- or dicholesterylphosphoric acid

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## THE RELATION OF SULFHYDRYL TO INHIBITION OF YEAST FERMENTATION BY IODOACETIC ACID

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The inhibiting effect of iodoacetic acid on tissue glycolysis and yeast fermentation has been suggested, by several investigators (1-3), to be due to its destructive action on the sulfhydryl system. If this explanation is correct, then the use of iodoacetic acid offers a means of attacking the general problem of the function of sulfhydryl in metabolic processes. While it has been shown (4) that certain proteolytic enzymes, for example cathepsin and papain, are activated by sulfhydryl, little is known of the part played by the latter in carbohydrate breakdown. Lohmann (5) has recently found that glyoxalase is specifically activated by glutathione. He believes, however, that this enzyme may not deserve the important place which has been ascribed to it in current theories on the mechanism of carbohydrate breakdown, and that glutathione probably does not play an essential rôle in zymase action. On the other hand, Barrenscheen and Beneschovsky (6) and Quastel and Wheatley (3) have presented evidence that sulfhydryl may be concerned in some of these reactions. The solution of this question is of considerable interest from the standpoint of cancer, since it is quite possible that a disturbance in the  $\text{—SH—SS—}$  equilibrium may be related in some way to the abnormally high glycolysis observed in these tissues. In fact, Bumm and Appel (7) have found that the addition of glutathione to tumor tissue increases the rate of aerobic glycolysis to the anaerobic level.

We have investigated the relation between inhibition of fermentation and loss of sulfhydryl in fresh bakers' yeast which has been treated with iodoacetic acid under various conditions. The

results obtained lead to the conclusion that the inhibiting effect of this substance cannot be attributed entirely to its action in destroying sulfhydryl. It is true that under certain conditions a marked parallelism exists between the inhibition and the loss of sulfhydryl, since (a) both effects are pronounced time reactions, (b) both effects vary with the pH in the same manner, being very rapid at 4.5 but decreasing as the pH increases, and (c) the relative effectiveness of iodoacetic,  $\alpha$ -, and  $\beta$ -iodopropionic acids in producing inhibition also represents roughly the reactivity of these substances towards pure glutathione. Closer examination of the quantitative relationships involved shows, however, that only a small part of the total sulfhydryl in the yeast is destroyed by the time inhibition is complete. With 5 per cent yeast suspensions and 1:5000 iodoacetic acid, this loss amounts in general to about 30 per cent or less. On the other hand, it is possible with free iodine to reduce the sulfhydryl content of yeast by as much as 70 per cent without obtaining complete inhibition. It is evident from this that, while a portion of the inhibition produced by iodoacetic acid may be due to its action on sulfhydryl, the larger part of its effect must be accounted for by some other reaction, probably by destruction of the zymase itself.

The effect of iodoacetic acid cannot be due to free iodine, these two substances differing considerably in their mode of action on yeast. Besides producing a much greater decrease in sulfhydryl for a corresponding amount of inhibition, iodine exerts its maximum effect almost immediately, and its action does not depend to any extent on the pH of the medium. Furthermore, yeast which has been only partially inhibited by small amounts of iodine undergoes a spontaneous reactivation with time, a behavior which is never observed with iodoacetic acid.

Comparison of the related compounds, iodoacetic,  $\alpha$ -, and  $\beta$ -iodopropionic acids, shows that the relative effectiveness of these substances in producing inhibition is roughly 200:20:1.  $\alpha$ -Iodopropionic acid closely resembles iodoacetic acid, complete inhibition being obtained when about 30 per cent of the sulfhydryl has been destroyed.  $\beta$ -Iodopropionic acid, on the other hand, despite its low reactivity towards pure glutathione, produces a much higher corresponding loss. In this respect it resembles free iodine, to the formation of which its action is believed to be in part due.

Experiments carried out with pure glutathione indicate that its reaction with iodoacetic acid does not involve oxidation of  $-\text{SH}$  to  $-\text{SS}-$ , or proceed through this mechanism to some other product. A more likely explanation would seem to be that of condensation of a molecule each of halogen acid and glutathione, with elimination of  $\text{HI}$ . Evidence in support of this view has recently been presented by Dickens (8).

#### EXPERIMENTAL

##### *Action of Iodoacetic Acid on Pure Glutathione*

*Effect of pH*—By means of the nitroprusside color test, it may easily be shown that iodoacetic acid reacts rapidly with sulfhydryl compounds, particularly in an alkaline medium. In order to determine quantitatively the effect of pH on the rate of this reaction, solutions were prepared containing 10 mg. of pure glutathione and 9 mg. of iodoacetic acid (1.5 mols per mol of GSH) in 50 cc. of 0.1 M phosphate buffer. The iodoacetic acid was neutralized with  $\text{NaOH}$  before use. The reaction mixtures were allowed to stand at room temperature. At intervals, 5 cc. portions were titrated for  $-\text{SH}$  by the iodate procedure of Woodward and Fry (9). Controls without iodoacetate were run at the same time to determine the autoxidation. In both cases, zinc reductions were carried out at intervals in order to determine the total oxidized and reduced glutathione present. The results are presented in Table I.

The marked effect of alkalinity on this reaction is shown by the fact that at pH 4.5 no loss of sulfhydryl occurs in 4 hours, while at pH 8.5 all has disappeared in less than 2 hours. A portion of this loss is due to autoxidation, as indicated by the controls. An interesting fact is revealed by the values obtained on reduction. In the controls, in which autoxidation of  $-\text{SH}$  to  $-\text{SS}-$  has occurred, the original  $-\text{SH}$  is completely recovered. On the other hand, in the samples containing iodoacetate, only a small portion of the amount lost is recovered by reduction. It is clear from this that the reaction between iodoacetic acid and glutathione is not simply an oxidation of  $-\text{SH}$  to  $-\text{SS}-$ .

*Effect of Iodoacetic Acid on  $-\text{SS}-$  Glutathione*—It was possible that the reaction might involve an oxidation of  $-\text{SH}$  through

—SS— to a product no longer reducible by zinc. To test this possibility, a solution of —SS— glutathione, prepared by aerating a solution of the reduced form at a slightly alkaline pH, was al-

TABLE I

*Effect of pH on Rate of Reaction between Iodoacetic Acid and Pure Glutathione*

pH	Time	GSH* present		GSH after zinc reduction	
		Iodoacetate	Control	Iodoacetate	Control
	hrs	per cent	per cent	per cent	per cent
4.5	1	100	100		
	2	100	100		
	4	99.4	99.6	100	100
6.4	1	93.9	96.0	95.3	100
	2	88.2	93.5		
	4	79.0	91.2	89.0	99.1
6.8	1	79.6	96.2	79.4	100
	2	62.4	91.2		
	4	42.2	83.0	54.3	98.8
8.5	1	9.4	77.0	23.6	98.8
	2	0	59.2		
	4			17.4	97.3

\* GSH represents reduced glutathione.

TABLE II

*Effect of Iodoacetic Acid on —SS— Glutathione*

Time	0.001 N iodate required after zinc reduction	
	Sample A	Sample B
hrs	cc	cc
0	1.10	1.85
1	1.19	1.80
2	1.19	1.92
21		2.06

lowed to stand at room temperature with excess iodoacetate in phosphate buffer of pH 8.5. At intervals, 10 cc. portions were removed, reduced with zinc as previously described, and titrated for sulfhydryl. As shown in Table II, no loss of reducible ma-

terial occurred in 21 hours, a slight tendency toward an increase being evident. Sample A contained 3.0 mols and Sample B 6.0 mols of iodoacetic acid per mol of oxidized glutathione.

Since no destruction of  $\text{—SS—}$  glutathione occurs, it must be concluded that the latter is not an intermediate in the reaction between iodoacetic acid and reduced glutathione. Bersin (1) has suggested a mechanism which involves elimination of HI and formation of a thio ether. The latter is then assumed to undergo oxidation to a sulfone, which acts as catalyst in the conversion of  $\text{—SH}$  to  $\text{—SS—}$  glutathione. It seems improbable from our results that the  $\text{—SS—}$  form is involved at all.

*$\alpha$ - and  $\beta$ -Iodopropionic Acids*—These two substances, despite their close structural relationship to iodoacetic acid, are much less reactive towards pure glutathione. With the same molar concentrations as were employed in the experiments with iodoacetic acid, it was found that  $\alpha$ -iodopropionic acid in 1 hour at pH 8.5 reacted with only 16.0 per cent of the glutathione originally present, as compared with 67.6 per cent for iodoacetic acid. These values were obtained by deducting from the total sulfhydryl lost the loss due to autoxidation as shown by the controls, and are based on the assumption that the rate of autoxidation was the same in the presence or absence of the halogen acid. At pH 4.5, no reaction occurred in 4 hours, as was the case also with iodoacetic acid. Another similarity is found in the fact that the reaction product of  $\alpha$ -iodopropionic acid with glutathione cannot be reduced to sulfhydryl by the usual zinc reduction method. It appears probable therefore that the mechanism of this reaction is similar to that of iodoacetic acid with glutathione.

$\beta$ -Iodopropionic acid on the other hand did not react with pure glutathione under the same conditions. The loss of sulfhydryl which occurred in 4 hours at pH 8.5 exactly paralleled that of the control, and was thus entirely accounted for by autoxidation. In phosphate buffers of pH 6.5 and 7.5, and with an excess of  $\beta$ -iodopropionic acid, 15 mols for each mol of glutathione, no reaction occurred on standing 24 hours at room temperature. The location of the halogen atom in the molecule therefore determines to a great extent the reactivity of these halogen acids toward glutathione.

*Relation between Loss of Sulfhydryl and Inhibition of  
Fermentation*

*Iodoacetic Acid*—It was shown in previous work (10) that the inhibition of yeast fermentation by iodoacetic acid is a pronounced time reaction depending on the pH of the medium. In the concentrations used, complete inhibition was obtained almost immediately at pH 4.5, while at 9.0 several hours elapsed before a noticeable effect was observed. Within this range the rate was intermediate. This dependence on external conditions, which has been attributed to changes in cell permeability, provides a means for comparing the rate of inhibition with the rate at which sulfhydryl disappears from treated yeast under different conditions of pH and concentration, and thus for determining what correlation exists between the two factors.

For the determination of sulfhydryl in yeast, an adaptation of the iodate method of Woodward and Fry (9) for blood glutathione was employed. Although there has been some question as to the accuracy of iodine methods for determining sulfhydryl in biological materials, this method was considered to be suited to the purpose. Satisfactory checks could be obtained on different portions of the same yeast sample, and pure glutathione added to yeast was quantitatively recovered. Qualitative experiments with the nitroprusside reagent showed that the development of color closely parallels the iodine consumption. The same observation has been made by Meldrum (11), who also used an iodine method for sulfhydryl in yeast. In tabulating our results, the iodine uptake was calculated in terms of reduced glutathione. On this basis, the glutathione content of fresh pressed bakers' yeast (Fleischmann) was found to be about 200 mg. per 100 gm. of yeast, the value varying slightly with different samples.

Reaction mixtures were made up so as to contain a 5 per cent suspension of the above yeast and 1:5000 iodoacetic acid (previously neutralized with NaOH) in 0.1 M phosphate buffers. After standing at room temperature for definite time intervals, 25 cc. portions were diluted to approximately 75 cc. with distilled water, centrifuged for 15 minutes, and the supernatant liquid was decanted. The residue was made up to exactly 25 cc. with water, 20 cc. were taken for the glutathione determination, and the re-

maining 5 cc. used for measuring the fermentative activity. To the 20 cc. portion were added 2 cc. of 22 per cent sulfosalicylic acid, and the mixture was boiled gently for 2 minutes, cooled, diluted to exactly 25 cc., and filtered. 10 cc. of the filtrate, corresponding to 0.4 gm. of yeast, were then titrated for sulfhydryl as previously described. The fermentative activity was determined by the manometric method described in the previous paper (10). The final concentrations in the fermentation vessels were 0.5 per cent yeast and 2 per cent glucose in a total volume of 2 cc. The volume of  $\text{CO}_2$  evolved in 20 minutes was taken as the measure of

TABLE III

*Inhibition of Fermentation and Loss of Sulfhydryl in Yeast Treated with Iodoacetic Acid*

The results are expressed in per cent.

Iodoacetic acid	pH	1 hr.		2 hrs		3 hrs		5 hrs		24 hrs	
		GSH lost	Inhibition	GSH lost	Inhibition	GSH lost	Inhibition	GSH lost	Inhibition	GSH lost	Inhibition
1:5000	4.5	48	100	63	100	71	100				
	5.0	27	98	32	100	50	100	61	100	86	100
	5.5	20	81	27	96	35	97	44	98	78	100
	6.0	16	43	22	71	26	94	28	98	78	100
	6.5	9	12	14	29	16	43	17	76	30	100
	7.5	0	0			0	0			5	32
	9.0	0	0			0	0			0	0
1:50,000	4.5	10	55	16	100	19	100	23	100	25	100
	5.0	5	7	7	24	9	50	11	88	18	100
	6.0	2	0	0	0	0	0	0	0	7	48

activity. The results are expressed in Table III in per cent of the original glutathione content and fermentative activity, as determined from controls.

It is evident that a parallelism exists between inhibition and loss of sulfhydryl. Both effects are pronounced time reactions, depending on the pH of the medium. The rate of loss of sulfhydryl is greatest at pH 4.5, where complete inhibition is obtained almost immediately with 1:5000 iodoacetic acid. As the pH is increased, these rates fall off, until at 7.5, no loss of sulfhydryl or inhibition of fermentation occurs in 3 hours. However, there appears to be no proportionality between these effects. In-



hibition is complete when only about 30 per cent of the total sulfhydryl has been destroyed. With 1:50,000 iodoacetic acid at pH 4.5, complete inhibition occurs when only 16 per cent of the sulfhydryl has disappeared. These experiments strongly indicate that the action of iodoacetic acid in inhibiting yeast fermentation cannot be accounted for, except to a relatively small degree, by its destructive action on sulfhydryl. In themselves, however, they do not wholly disprove the latter hypothesis, since it would seem quite possible that only a slight destruction of sulfhydryl, sufficient to disturb the  $\text{—SH—SS—}$  equilibrium, might be enough to produce complete inhibition. That such is not the case is indicated by experiments described in a succeeding section, on the effect of free iodine on yeast fermentation.

An additional fact to which attention should be called is revealed by the experiments with 1:50,000 iodoacetic acid. This concentration represents only 30 per cent of the theoretical amount necessary to react mol for mol with the glutathione present, the latter being calculated from the iodine uptake. Examination of Table III shows that with this concentration at pH 4.5, 25 per cent of the total glutathione, thus very nearly the theoretical amount, was destroyed in 24 hours. This is evidence that the loss of glutathione is due to direct reaction with iodoacetic acid, and is not a secondary effect. In a recent paper, Barrenscheen and Beneschovsky (6) reported that when iodoacetic acid is injected into the lymph sac of a frog, muscle rigidity occurs, and all of the  $\text{—SH}$  glutathione in these muscles is converted into the  $\text{—SS—}$  form. This is probably a secondary effect, and not due to direct action of iodoacetic acid on  $\text{—SH}$  glutathione, since our experiments indicate that this reaction does not lead to the  $\text{—SS—}$  form.

*$\alpha$ - and  $\beta$ -Iodopropionic Acids*—A series of experiments was carried out by the manometric method to determine the relative effectiveness of these compounds, as compared to iodoacetic acid, in producing inhibition of fermentation. The glucose, buffer, and halogen acid (previously neutralized with NaOH) were placed in the main chamber and the yeast suspension in the side arm of the manometric vessel. After saturation with  $\text{CO}_2$ , the yeast was washed into the main chamber, the final concentrations in a volume of 2 cc. being 0.5 per cent yeast, 2 per cent glucose, and 0.1 M

phosphate buffer of pH 4.5. By varying the concentration of halogen acid, it was possible to determine the smallest amount which would produce complete inhibition at the end of 20 minutes after the start of fermentation. It was found that these concentrations, in parts by weight in the 2 cc. volume, were 1:50,000, 1:5000, and 1:250, for iodoacetic,  $\alpha$ -, and  $\beta$ -iodopropionic acids, respectively, the relative effectiveness in producing inhibition therefore being 200:20:1, in the order given.

In view of the fact that the iodopropionic acids were considerably less reactive towards pure glutathione than was iodoacetic acid, it was of interest to study their effect on the glutathione con-

TABLE IV

*Inhibition of Fermentation and Loss of Sulphydryl in Yeast Treated with  $\alpha$ - and  $\beta$ -Iodopropionic Acids*

The results are expressed in per cent.

Acid	pH	1 hr		4 hrs		23 hrs		54 hrs	
		GSH lost	Inhibition	GSH lost	Inhibition	GSH lost	Inhibition	GSH lost	Inhibition
$\alpha$ -Iodopropionic, 1:5000	5.0	2	3	12	60	42	100		
	6.5	0	0	0	0	2			
$\beta$ -Iodopropionic, 1:1000	5.0	4	3	13	30	34	54	48	76
	6.5	0	0	0	9	6	12		12

tent of yeast. The results of these experiments, which were carried out in exactly the same manner as those with iodoacetic acid, are presented in Table IV. The effect of  $\alpha$ -iodopropionic acid is quite similar to that of iodoacetic acid, but much slower. In 4 hours at pH 5.0, only 12 per cent loss of glutathione occurs as compared to about 55 per cent with iodoacetic acid. The fact that 60 per cent inhibition was obtained when only 12 per cent of the glutathione had been destroyed indicates that probably not more than 30 per cent loss had occurred by the time inhibition was complete. This corresponds closely to the value obtained with iodoacetic acid.

$\beta$ -Iodopropionic acid, on the other hand, shows an unexpected behavior. Although a 1:1000 concentration of this acid does not react with pure glutathione in 24 hours even at pH 7.5, it shows considerable reactivity towards the glutathione in yeast, 34 per

cent of the total being destroyed in 23 hours and 48 per cent in 54 hours. An interesting fact is that, despite these high losses, the fermentation is inhibited only 54 per cent and 76 per cent respectively. This corresponds closely to the effect produced by free iodine, and is no doubt to be attributed, at least in part, to the presence of this substance.  $\beta$ -Iodopropionic acid yields HI by hydrolysis much more readily than does either iodoacetic or  $\alpha$ -iodopropionic acid (12), and the formation of free iodine could thus be accounted for by the presence of an oxidizing agent in the yeast.

*Iodine*—That the effect of iodoacetic acid is not due to free iodine is indicated by the fact that these substances differ considerably in their mode of action on yeast fermentation. When varying quantities of iodine<sup>1</sup> are added to a yeast suspension, the effect is almost immediate, the maximum inhibition being secured in less than 15 minutes, after which no further increase in inhibition occurs. In fact, it was found that in those samples which were only partially inhibited, a spontaneous reactivation occurred with time. Thus, samples which were 50 per cent inhibited at the start of the fermentation, increased to the control value in the course of several hours. On the other hand, samples which were completely inhibited at the start, rarely showed any reactivation. It will be recalled that with iodoacetic acid, inhibition always increased with time, and no reactivation took place. Also, in contrast to the results obtained with iodoacetic acid, the pH, within the range studied (4.5 to 7.0), had no appreciable effect on the amount of inhibition obtained with iodine. It is probable that the yeast cell is permeable to iodine over this pH range, and once inside, its effect is exerted at once.

Table V shows the relation between the degree of inhibition and loss of sulfhydryl produced when varying amounts of iodine<sup>1</sup> were added to yeast suspensions. The same general procedure was employed in these experiments as in the case of iodoacetic acid. 2 per cent suspensions of yeast, containing varying amounts of iodine, were allowed to stand at room temperature for 30 minutes, centrifuged, made up to volume, and the glutathione and fermentative activity determined as previously described. Separate ex-

<sup>1</sup> The stock iodine solution used was 0.001 N, in 0.025 per cent KI. Separate experiments showed that the final concentrations of KI used (1.115,000 to 1.115,500) had no effect on fermentation.

periments showed that the results were not changed by increasing the time of standing to 1 hour. The concentrations of iodine used are expressed in parts by weight and in equivalents of iodine per mol of glutathione present in the 2 per cent suspension.

While with iodoacetic acid only 30 per cent or less of the total glutathione is destroyed by the time inhibition is complete, with iodine a much larger proportion may be destroyed without completely stopping fermentation. It is evident, therefore, that destruction of a small part of the total sulfhydryl, or merely disturbing the ratio of  $-SH$  to  $-SS-$  is not sufficient to interrupt the activity of the yeast entirely.

TABLE V  
*Effect of Iodine on Fermentation and Sulfhydryl Content of Yeast*

Iodine concentration (by weight)	Equivalents Iodine GSH	Decrease in	
		Fermentation	Sulfhydryl
		<i>per cent</i>	<i>per cent</i>
1 225,000	1 1	22	18
1 112,000	2 1	49	39
1 75,000	3 1	66	49
1 56,000	4 1	92	70
1 45,000	5 1	100	94

#### DISCUSSION

These results, while indicating that other factors besides destruction of glutathione are involved in the inhibitory action of iodoacetic acid on yeast, do not necessarily mean that sulfhydryl compounds are not concerned in the processes of fermentation. In fact, the close parallelism between loss of sulfhydryl and inhibition in the case of iodine-treated yeast would suggest that there is a close connection. The interesting observations of Quastel and Wheatley (3) on the stimulating action of glutathione and cysteine on aerobic fermentation point to the same conclusion. It is probable that, with iodoacetic acid, other effects outweigh its action on the sulfhydryl compounds, so that the influence of the latter is not apparent. We have thus far not observed any effect of glutathione on fermentation under anaerobic conditions. Nor have we been able to reactivate yeast, which has been par-

tially or wholly poisoned with iodine or iodoacetic acid, by adding glutathione. This is further evidence that some other essential cell constituent has been destroyed by these substances. Further investigation is necessary to determine the exact mechanism of their action. It would also be desirable to extend these experiments to animal tissue, and to determine what relation exists in this case between loss of sulfhydryl and inhibition of glycolysis.

#### SUMMARY

Glutathione measurements in yeast whose fermentation has been partially or wholly inhibited by iodoacetic acid show that a decrease in glutathione content runs parallel with the degree of inhibition, but that inhibition is complete when 30 per cent or less of the glutathione has been lost. This is true also with  $\alpha$ -iodopropionic acid, but not with  $\beta$ -iodopropionic acid or free iodine. With iodine, the glutathione content may be reduced by more than 70 per cent before inhibition is complete. This shows that the inhibitory action of iodoacetic acid on yeast fermentation cannot be attributed entirely to its action in destroying glutathione.

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## INTERMEDIARY COMPOUNDS IN THE ACETONE-BUTYL ALCOHOL FERMENTATION\*

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Numerous compounds such as methylglyoxal, acetaldehyde, acetoacetic acid, aldol, and  $\beta$ -hydroxybutyric acid have been postulated as intermediates in the breaking down of carbohydrates by the acetone-butyl alcohol organism, *Clostridium acetobutylicum* (1).

The most direct method of establishing the existence of intermediary products is to isolate them from the fermenting solution. This is rarely possible because of the fleeting existence of such compounds and the extremely small quantity present at any particular moment. Another valuable but less convincing procedure is to add the assumed intermediate to the medium and, if it proves fermentable, to determine the products formed. In the present paper both of these lines of investigation have been followed.

### EXPERIMENTAL

*Medium and Culturing*—The medium used was the same as that described in a previous communication (2). The compound, the fermentability of which was under investigation, was added to this medium, which was then inoculated with a vigorously fermenting culture of *Clostridium acetobutylicum* in corn mash. 1 per cent of inoculum was used. The cultures were incubated at 37°.

*General Analytical Methods*—Glucose was determined in the medium before and after fermentation by the procedure of Stiles, Peterson, and Fred (3). For the determination of fermentation products, 50 cc. of fermented culture were placed in a flask con-

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nected to an efficient condenser, made very slightly alkaline, and distilled until about 10 cc. were left. The distillate, collected in a 50 cc. volumetric flask, was diluted to 50 cc. and aliquots of it were taken for the iodometric determination of acetone and for the determination of alcohol by a previously published method (4). The 10 cc. of residue, acidified with a few drops of concentrated sulfuric acid, were steam-distilled at constant volume and 110 cc. were collected in a volumetric flask. Of the distillate, 100 cc. were subjected to a Duclaux distillation as modified by Virtanen and Pulkki (5). Their method was modified somewhat in that the volume taken for distillation and the volumes of the distillate fractions were reduced by one-half. When pyruvic acid was present in the culture, two successive steam distillations were necessary because pyruvic acid is somewhat volatile. The first distillate was neutralized, boiled down to 10 cc., reacidified, and again steam-distilled.

*Determination of Acetylmethylcarbinol*—Acetylmethylcarbinol was estimated by the procedure described by Wilson, Peterson, and Fred (6). This method gives recoveries of about 72 per cent when either acetylmethylcarbinol or diacetyl is employed as starting material, and this recovery was assumed in calculating the results. When acetylmethylcarbinol was determined, the glucose, acetone, and ethyl alcohol determinations were corrected for the errors due to its presence by use of experimentally determined values for the volatility of acetylmethylcarbinol. It was found that in alkaline solutions 1 molecule of acetylmethylcarbinol reacts with 6 atoms of iodine. The acetone determinations were corrected by an amount calculated from this relationship. Under the conditions of the alcohol oxidation procedure, acetylmethylcarbinol is oxidized to 2 molecules of acetic acid. The ethyl alcohol determinations were corrected accordingly.

*Determination of Acetoacetic Acid*—For the determination of acetoacetic acid in cultures, the method found most convenient and accurate is here described.

The sample of culture (10 to 50 cc.) was placed in a cylinder or large test-tube, saturated with sodium chloride, and aspirated for 1 hour with a vigorous current of air to drive off all the acetone. A few drops of paraffin oil were added to prevent frothing. The sample was then diluted with an equal volume of water and dis-

tilled. Acetoacetic acid, if present, decomposed to form acetone, which was determined iodometrically in the distillate. If the quantity of acetoacetic acid is very small, this method offers a serious difficulty. Acetylmethylcarbinol, which is always present in the culture, is somewhat volatile, and because it reacts with alkaline iodine solution, interferes with the determination. The difficulty could not be overcome by determining the acetone by a precipitation method, such as that of Scott-Wilson (7), since acetylmethylcarbinol gave a precipitate with the reagent.

*Determination of Pyruvic Acid*—In order to investigate quantitatively the fermentation of pyruvic acid, an accurate method of determination was necessary. The method of Kendall and Friedemann (8), according to the authors, gives recoveries ranging from 80 to 94 per cent. For the construction of fermentation balances, however, a somewhat higher degree of accuracy is necessary. The method to be described has been found to be rapid and accurate. It has not, however, been tested upon materials other than cultures of the acetone-butyl alcohol organism, and therefore is not offered as being generally applicable. It depends upon the fact that precipitated manganese dioxide, in the presence of 15 per cent sulfuric acid, will, at room temperature, quantitatively oxidize pyruvic acid to acetic acid and carbon dioxide. The carbon dioxide evolved is determined volumetrically.

A sample of culture is acidified with sulfuric acid, and extracted with ether in a continuous extractor. Water is added to the ether extract, and the ether is gently boiled off. This aqueous solution, or an aliquot of it containing 20 to 80 mg. of pyruvic acid, is diluted to 20 cc., and 2 cc. of concentrated sulfuric acid and 5 cc. of an approximately 9 per cent suspension of precipitated  $\text{MnO}_2$  are added. A slow current of  $\text{CO}_2$ -free air is bubbled through the reaction mixture for 45 minutes, during which time the containing vessel (a large test-tube) is shaken occasionally.

The  $\text{CO}_2$  given off is absorbed from the air stream and determined by any standard method. That of Heck (9) has been found very satisfactory. Formic acid yields a small amount of carbon dioxide by this procedure, but fortunately only small amounts of this compound are present in the culture, so that an empirical correction can be satisfactorily made. The recovery of pyruvic acid added to culture medium, and determined by this method, was 98 to 101 per cent.



*Acetoacetic Acid*

All investigators have assumed that acetoacetic acid is the immediate precursor of acetone in the fermentation. The only evidence for this view has been the fact that Reilly *et al.* (10), and Speakman (11) have found that added acetic acid is transformed into acetone, 1 molecule of acetone being formed from 2 molecules of acetic acid. The simplest manner of representing such a transformation is by postulation of acetoacetic acid as an intermediate product.

In the present investigation, many attempts were made to demonstrate the presence of acetoacetic acid in the normal culture. Color tests were either inconclusive or negative while iodometric determinations showed but little more iodine consumption than could be accounted for by the acetylmethylcarbinol which was present. The uniform negativity of the various tests and analytical procedures seemed to demonstrate that if acetoacetic acid was present at all, it occurred only in extremely small amounts.

*Decarboxylation of Acetoacetic Acid by Normal Culture*—The apparent absence of this compound from the culture seemed to indicate that the organism possessed the power of rapidly decarboxylating it. In order to determine whether such was the case, some preliminary experiments were performed in which acetoacetic acid, in the form of its sodium salt, was added to the culture. In every case all the acetoacetic acid was destroyed within a few hours and an equivalent quantity of acetone was formed. Control flasks, containing sodium acetoacetate in a buffer solution showed, in the same time, but little decarboxylation. It was necessary, however, to perform all decarboxylation experiments at 25° rather than at 37°, since at the latter temperature spontaneous decarboxylation of acetoacetic acid occurs at a very appreciable rate. Data for three such experiments are given in Table I. The recovery of destroyed acetoacetic acid ranged from 98 to 109 per cent.

In the next series, cultures of different age were tested and the concentration of acid and period of incubation were varied. The results are given in Table II. In Table II and elsewhere in this paper, the acetoacetic acid concentrations are expressed in terms of the equivalent quantity of acetone. From the data it will be seen that the rate of decomposition varied with different cultures

but that the decarboxylation behaved approximately as a monomolecular reaction. In the case of Culture 3, for example,  $k$  is approximately constant for decarboxylation periods of different

TABLE I  
*Formation of Acetone from Acetoacetic Acid by Cultures*

Age of culture	Incubation time	Acetoacetic acid*			Acetone formed in culture	Recovery of destroyed acid, as acetone
		Added	Decomposed in culture	Decomposed in control		
hrs	hrs	gm per l	gm per l	gm per l	gm per l	per cent
36	4	1 92	1 21	0 19	1 19	98
36	8	1 92	1 83	0 19	1 89	103
38	8	3 60	2 61	0 24	2 84	109

\* Expressed in terms of the equivalent quantity of acetone.

TABLE II  
*Rate of Destruction of Acetoacetic Acid by Different Cultures*

Culture No	Age of culture	Period of decarboxylation (t)	Acetoacetic acid*		Velocity constant, $k$ $\left(\frac{1}{t} \log_{10} \frac{a}{a-x}\right)$
			Beginning (a)	Ending (a-x)†	
	hrs	hrs	gm per l.	gm. per l	
1	38	9	3 60	1 23	0 052
2	39	11	1 92	0 86	0 032
3	54	1	4 67	3 70	0 102
3	54	1	4 67	3 78	0 093
3	54	4	4 67	2 07	0 090
3	54	4	4 67	2 14	0 085
3	54	9	4 67	0 53	0 105
3	54	9	4 67	0 58	0 101

\* Expressed in terms of the equivalent quantity of acetone.

† Concentration in fermenting culture plus spontaneous decomposition in control. The latter amounted to from 1 to 5 per cent of the initial concentration, depending on the period of incubation.

lengths. Variation of decarboxylating ability with age was determined by taking 400 cc. samples from a 5 liter culture at intervals during the fermentation. To each of these samples, sodium acetoacetate equivalent to 2 gm. per liter of acetoacetic acid was added and after 4 hours incubation at 25° analyses were made.

Acetoacetic acid-fermenting ability together with data on the acidity and acetone concentration is plotted against time in Fig. 1. The ability to ferment acetoacetic acid is expressed in terms of  $k$ , which has the same significance as in Table II. The graphs representing acetoacetic acid-fermenting ability and acetone production are approximately parallel for the first 40 hours, and indicate the close relationship existing between the two phenomena.

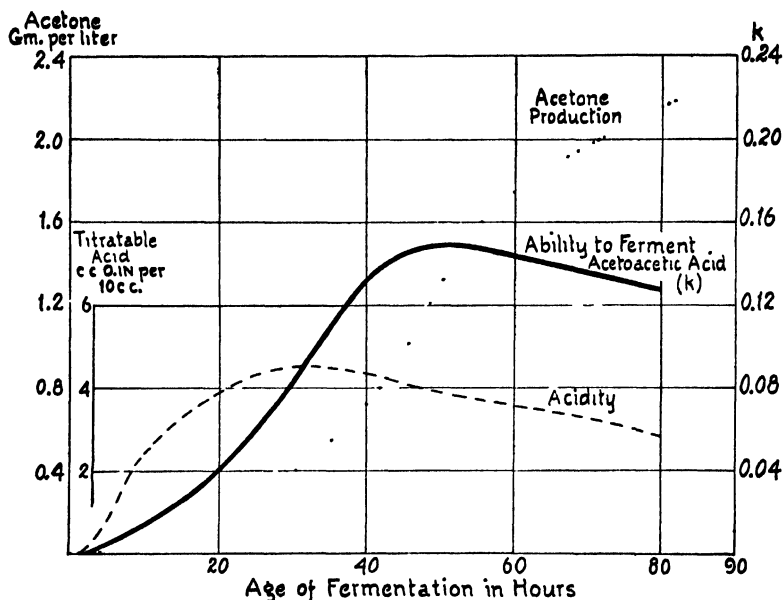


FIG. 1 Effect of age on ability of culture to ferment acetoacetic acid

The period of greatest acetoacetic acid-fermenting ability is also the period of most rapid acetone production. It should be noted that the velocity constant,  $k$ , was determined at 25°, whereas the fermentation itself took place at 37°. The rate of destruction of acetoacetic acid is undoubtedly much greater at 37° than at 25°, since acetoacetic acid is very unstable at higher temperatures. If, however, we neglect this temperature effect, and if we assume that the decarboxylation of acetoacetic acid is a monomolecular reaction, we are able to calculate a value for the acetoacetic acid concentration in the fermenting culture from the data of Fig. 1. Such a calculation for the period when  $k$  is approximately constant,

45 to 65 hours, yields a value in the neighborhood of 0.3 gm. per liter. While the actual concentration is probably much smaller than this, it seems entirely possible that acetoacetic acid would be detectable in the culture were it not for the fact that during the long aspiration period (5 hours) necessary to free a large volume of culture from acetone, acetoacetic acid is being decarboxylated but not formed.

TABLE III

*Effect of pH and Antiseptics on Decarboxylation of Acetoacetic Acid by Cell Suspensions*

Treatment of cell suspension	Incubation period	pH at beginning	pH at end	Acetoacetic acid at end*	Acid destroyed
	hrs			gm per l	per cent
None	17	6.8	8.0	1.22	37
"	17	6.8	8.1	0.94	52
Phosphate (KH <sub>2</sub> PO <sub>4</sub> , 2 per cent)	6	5.2	6.4	0.45	77
" heated to boiling and cooled	6	5.2		1.88	4
Phosphate and ether	6	5.2		0.49	75
" " toluene	6	5.2		0.47	76
" " salicylic acid (0.5 per cent)	6	5.2		0.72	63
Control	24	7.0	7.1	1.83	6
"	24	5.0	5.1	1.80	8

Cells, 5 gm. per liter (wet basis); acetoacetic acid at beginning, 1.95 gm. per liter; temperature of incubating, 25°. Control contained phosphate and acid but no cells.

\* Expressed in terms of the equivalent quantity of acetone.

*Decarboxylation of Acetoacetic Acid by Centrifuged Cells*—Cells centrifuged from a vigorously fermenting culture of *Clostridium acetobutylicum* and washed, neither fermented glucose nor reduced methylene blue in the presence of glucose. The reason for this inactivity probably lies in the toxicity of oxygen to the organism. However, the cell suspension possessed almost undiminished its power of decarboxylating acetoacetic acid. The reaction must, however, be carried on in the presence of a buffer, otherwise the sodium hydroxide liberated from the sodium acetoacetate soon raises the pH to a value at which decarboxylation ceases. This inhibition of activity by alkali is brought out by Table III, which

shows that more decarboxylation occurred in 6 hours in the presence of a buffer than occurred in 17 hours without it.

The effect of heat, toluene, ether, and salicylic acid upon the decarboxylation is also shown in Table III. The antiseptics have but little effect, while heat destroys the active agent completely.

The activity of the cell suspension was also tested on two acids closely related to acetoacetic acid, *i.e.* malonic and pyruvic, but neither of these acids was decarboxylated. Since the resting (washed) cells could decarboxylate acetoacetic acid, the question naturally arose as to whether the cell-free medium could also bring about this change. An actively fermenting culture was passed through a Berkefeld filter and the filtrate was tested for decarboxylating ability but it did not act upon acetoacetic acid. It follows, therefore, that the agent responsible for the decarboxylation is to be found only in the cell.

#### *$\beta$ -Hydroxybutyric Acid*

The precursor of acetoacetic acid, according to Donker, is acetic acid, but according to Schoen (12) it is  $\beta$ -hydroxybutyric acid. Although it is difficult to reconcile Schoen's fermentation mechanism with the experimental fact that acetic acid added to a fermentation is transformed almost entirely into acetone, it was thought worth while to investigate the fermentability of  $\beta$ -hydroxybutyric acid. Two flasks, each containing 700 cc. of 1 per cent glucose medium and an excess of calcium carbonate, were inoculated. To one flask, before inoculation, was added 1 per cent of synthetic  $\beta$ -hydroxybutyric acid as the sodium salt prepared according to the method of Wislicenus (13). Upon completion of the fermentation both flasks were analyzed for fermentation products. Data are given in Table IV. A good fermentation was secured in each case; but in the flask containing added  $\beta$ -hydroxybutyric acid acetyl-methylcarbinol production was largely inhibited. It will be noted that in both flasks the products formed are fully accounted for by the quantity of glucose which disappeared.<sup>1</sup> It follows, of course,

<sup>1</sup> Some explanation should perhaps be made concerning the method used in this paper of balancing products and fermented glucose. In the column marked "Glucose equivalent" are given mm of glucose theoretically equivalent to the mm of each product formed. Thus, since 2 molecules of acetic acid arise from 1 molecule of glucose, 20.8 mm of acetic acid have a glucose equivalent of 10.4 mm.

that none of the  $\beta$ -hydroxybutyric acid underwent fermentation. This method of testing the fermentability of  $\beta$ -hydroxybutyric acid was thought to be preferable to a determination of the quantity of the compound remaining after fermentation, since consistent results could not be obtained in the determination.

An attempt was also made to isolate  $\beta$ -hydroxybutyric acid from a normal culture. The non-volatile, ether-soluble acids from 100 liters of culture were isolated in the form of their zinc salts. The most soluble fraction of the mixed zinc salts was reextracted, and

TABLE IV

*Products of Normal Fermentation and of One Containing  $\beta$ -Hydroxybutyric Acid*

Product	Flask I		Flask II	
	Quantity produced	Glucose equivalent*	Quantity produced	Glucose equivalent*
	mm per l	mm per l	mm per l	mm per l
Butyric acid	26 5	26 5	29 4	29 4
Butyl alcohol	10 6	10 6	5 2	5 2
Acetic acid	20 8	10 4	26 9	13 5
Acetone	0 8	0 8	1 5	1 5
Ethyl alcohol	3 0	1 5	3 4	1 7
Acetylmethylcarbinol	4 4	4 4	0 2	0 2
Total equivalent glucose		54 2		51 5
Glucose fermented		55 1		53 0

Flask I contained 1 per cent glucose; Flask II contained 1 per cent glucose plus 1 per cent  $\beta$ -hydroxybutyric acid. An excess of calcium carbonate was present in both flasks.

\* For method of calculating "Glucose equivalent" see foot-note 1.

the mixed calcium-zinc salt prepared. Shaffer and Marriott (14) have found that calcium-zinc- $\beta$ -hydroxybutyrate is relatively insoluble. No  $\beta$ -hydroxybutyric acid could be isolated. It seems likely, from experiments with synthetic  $\beta$ -hydroxybutyric acid, that if as much as 0.5 gm. of the substance had been present in the 100 liters of culture, its presence would have been detected.

#### *Fermentation of Pyruvic Acid*

Pyruvic acid has been mentioned as a possible intermediate in the butyl fermentation, and it was thought that an investigation of its

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fermentability might throw some light on the rôle of this compound, or of acetaldehyde, in the fermentation. After preliminary experiments had shown that pyruvic acid was easily fermented, the method described above was devised for its estimation, so that detailed fermentation analyses might be made. In Table V are summarized the results obtained from two fermentations, to one of which 0.75 per cent of freshly distilled pyruvic acid, together

TABLE V  
*Fermentation of Glucose, and of Glucose Plus Pyruvic Acid*

Product	Flask I		Flask II	
	Quantity of product	Glucose equivalent*	Quantity of product	Glucose equivalent*
	<i>mm per l</i>	<i>mm per l</i>	<i>mm per l</i>	<i>mm per l</i>
Butyric acid	26 0	26 0	33 0	33 0
Butyl alcohol	8 2	8 2	6 0	6 0
Acetic acid ..	21 1	10 5	79 4	39 7
Acetone	1 2	1 2	5 6	5 6
Ethyl alcohol	2 8	1 4	2 6	1 3
Acetylmethylcarbinol	2 7	2 7	5 4	5 4
Total products		50 0		91 0
Fermented glucose	53 2	53 2	52 1	52 1
“ pyruvic acid	0 0		80 4	40 2
Total fermented compounds		53 2		92 3

Flasks I and II contained 56.3 mm per liter of glucose plus 0.425 per cent of calcium carbonate. Flask II contained in addition 85.2 mm per liter of pyruvic acid. 400 cc. of medium were used in each flask.

\* For method of calculating "Glucose equivalent" see foot-note 1.

with a quantity of calcium carbonate sufficient to neutralize the pyruvic acid, was added before sterilization and inoculation. An equal quantity of calcium carbonate was added to the control flask. The data show that 94 per cent of the added pyruvic acid was fermented, and that the glucose and pyruvic acid fermented are accounted for by the products formed. It will also be noticed that while the addition of pyruvic acid greatly increased the total quantity of products, there was but little increase in the production of 4-carbon compounds, *i.e.* butyl alcohol and butyric acid. Acetylmethylcarbinol is not counted as a 4-carbon compound

since its synthesis is undoubtedly accomplished in a different manner. In the control fermentation 0.64 mol of 4-carbon compounds was produced per mol of sugar fermented, while in the fermentation containing pyruvic acid the figure was 0.75. At the same time, the remaining compounds (calculated on a 2-carbon basis)<sup>2</sup> increased from 0.60 to 2.00 mols per mol of sugar fermented. Another pyruvic acid fermentation, upon which acetylmethylcarbinol was not determined, and to whose control calcium carbonate was not added, gave an increase in 4-carbon compounds from 0.61 to 0.74 mol, and an increase in other compounds from 0.79 to 2.16 mols. It appears that most, but not all, of the pyruvic acid fermented was transformed into acetic acid, acetone, and acetylmethylcarbinol.

The balance secured between compounds fermented and compounds produced shows that 1 of the carbon atoms of pyruvic acid is lost as  $\text{CO}_2$ , the other 2 appearing as products analyzed. The fate of the greater portion of the fermented pyruvic acid is easily traced. The observed products of its breakdown are acetic acid, acetone, and acetylmethylcarbinol. The increased acetylmethylcarbinol production is evidence that acetaldehyde was formed; the increased acetic acid production shows that this acetaldehyde was dehydrogenated. The increased yield of acetone implies that a part of the acetic acid formed was condensed to acetoacetic acid, and decarboxylated. The explanation of the fact that only small amounts of 4-carbon compounds are formed from pyruvic acid lies in its degree of oxidation. Pyruvic acid contains 2 less hydrogen atoms than a triose. Since production of butyric acid and butyl alcohol involves reduction reactions, a scarcity of hydrogen available for reduction reactions results in inhibition of their formation. Ample support for this line of reasoning is to be found in the difference in products observed when mannitol, glucose, and gluconic acid are fermented (2).

#### *Non-Fermentability of Methylglyoxal and Aldol*

Numerous attempts were made to bring about the fermentation of methylglyoxal by the organism. It was found, however, that this compound, even when added to the extent of only 0.03 per

<sup>2</sup> In calculation on a 2-carbon basis the total number of mols produced is taken to be the mols of ethyl alcohol plus the mols of acetic acid plus twice the mols of acetone plus twice the mols of acetylmethylcarbinol.



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cent, quickly inhibited a previously vigorous fermentation. Pett and Wynne (15), however, have found that a dry cell preparation of *Clostridium acetobutylicum* is able to transform hexosediphosphate into methylglyoxal. Whereas the ordinary form of methylglyoxal was not fermentable, it may be that an active form is produced by the organism, and is readily fermented. This may also be true of aldol which Donker and others have postulated as an intermediate but which we found to be, in its ordinary form, toxic to the organism.

### SUMMARY

The occurrence of certain assumed intermediates in the fermentation of carbohydrates by the acetone-butyl alcohol organism, *Clostridium acetobutylicum*, has been investigated by two procedures: (1) attempted isolation of the compound, (2) determination of its fermentability.

Acetoacetic acid when added to a vigorously fermenting culture was rapidly decarboxylated to acetone. The ability of the organism to ferment acetoacetic acid reached a maximum at the time of most rapid acetone production. Decarboxylation of the acid was also accomplished by centrifuged and washed cells of *Clostridium acetobutylicum*. The decarboxylating agent was not contained in a Berkefeld filtrate of the culture and hence appears to be intracellular.

Pyruvic acid was readily fermented by the organism. It was transformed mainly into acetic acid, acetone, and acetylmethylcarbinol. A new method for the estimation of pyruvic acid has been developed.

$\beta$ -Hydroxybutyric acid, when added to a vigorous fermentation of glucose, was apparently not destroyed. However, it did not interfere with the continued progress of the fermentation. Also attempts to isolate this acid from a normal fermentation failed.

Methylglyoxal and aldol, even in small amounts, proved toxic to the organism.

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## THE BIOLOGICAL ACTION OF STRONGLY POSITIVE OXIDATION-REDUCTION SYSTEMS

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The intermediate metabolism of proteins gives rise to hydroxylated phenols from the tyrosine and phenylalanine radicals. These are potential oxidation-reduction systems circulating in the blood, and, as will be proved later, extremely positive from a biological point of view.

A survey of the physiological oxidation-reduction systems identified up to the present shows that they have previously been roughly divided into two classes: a more negative, such as cysteine, glutathione, sugar, echinochrome, hermidine, etc., and a more positive, such as hemoglobin-methemoglobin, cytochrome, and Warburg's respiratory ferment. With the exception of the work of Conant and Fieser (1) on hemoglobin and of Ball and Chen on very positive systems (2), the potential of the more positive systems has not been well determined. The cyclic products of protein metabolism mentioned above must now be considered in their relation to other systems in the organism. Their physiological action can only be really appreciated by a study of their pathological manifestations, since the excellent poisoning effect of the systems of the middle range, such as hemoglobin, makes them imperceptible. Pathologically the polyhydroxylated phenols make their appearance in two conditions. The first is congenital; an inherited metabolic anomaly, a total inability to destroy phenylalanine and tyrosine, results in the excretion of homogentisic acid as an end-product in the urine, and after many years, the deposition of a black pigment, almost entirely limited to the cartilage and sclerae. Carbolochronosis (3) is a similar condition in which the application of phenol over a long period of years results in the deposition of an exactly similar pigmentation. Secondly, there are

the cases of poisoning by acetanilide, resorcinol, aniline, nitrobenzene, and similar aromatic substances. Of late years with the development of the aniline dye industry this has become a problem of major importance. The formation of methemoglobin by metabolic products of the pneumococcus, with consequent diminution in the oxygen capacity of the blood in pneumonia patients (Stadie (4)), may perhaps also be included in this list. As demonstrated by Pick (5) for ochronosis, the common etiological factor in all these conditions is the circulation of polyhydroxylated phenols and their oxidation products in the blood.

Homogentisic acid is chemically 1,4-*p*-dihydroxyphenylacetic acid with the side chain ortho to one of the hydroxyl groups. It was isolated from the urine of a patient<sup>1</sup> suffering from alkaptonuria by acidifying a 24 hour specimen of urine with 250 cc. of 12 per cent  $\text{H}_2\text{SO}_4$  and extracting the acidified urine three times with an equal amount of ether. On evaporation of the extract the dark brown syrupy residue is dissolved in 250 cc. of boiling water. 30 cc. of 20 per cent lead acetate are added and filtered through a fluted filter. On standing, the lead salt of homogentisic acid precipitates. This is filtered, washed with a minimum of ice water, redissolved,  $\text{H}_2\text{S}$  is passed in, and the lead precipitated out as sulfide. On evaporating the filtrate *in vacuo*, and on recrystallizing from boiling water, a pure product (m.p.  $145^\circ$ ) is obtained.

The system homogentisic acid-benzoquinone acetic acid fulfils all criteria of a reversible oxidation system.

As is to be seen from Chart 1 and Table I, a typical curve is obtained on oxidation of the reduced phase with stronger oxidizing agents. This was titrated at various pH values between 0.6 and 2.7 with potassium dichromate of the same pH without special precautions for the exclusion of air; also between pH 5.5 and 7.2 with potassium ferricyanide in an atmosphere of nitrogen. (For full details of the technique see Clark *et al.* (6).) Reduction of the oxidized form with titanous chloride prepared according to Clark gave analogous results.

The insertion of bright platinum electrodes into equimolecular, heavily buffered mixtures of the reduced and oxidized phases

<sup>1</sup> We wish to express our thanks to Dr. S. R. Benedict for rendering this work possible by suggesting an alkaptonuric patient to us.

gives sharp potentials as shown in Chart 2 and Table II. Also the potentials obtained are numerically equal to those of the mid-points of the oxidative and reductive titrations.

The form of Chart 2, which is that of a modified bayonet, could have been predicted from a study of the chemical constitution of the molecule. This molecule contains 3 dissociable hydrogen atoms, of which 1 can split off from the carboxyl group at a much

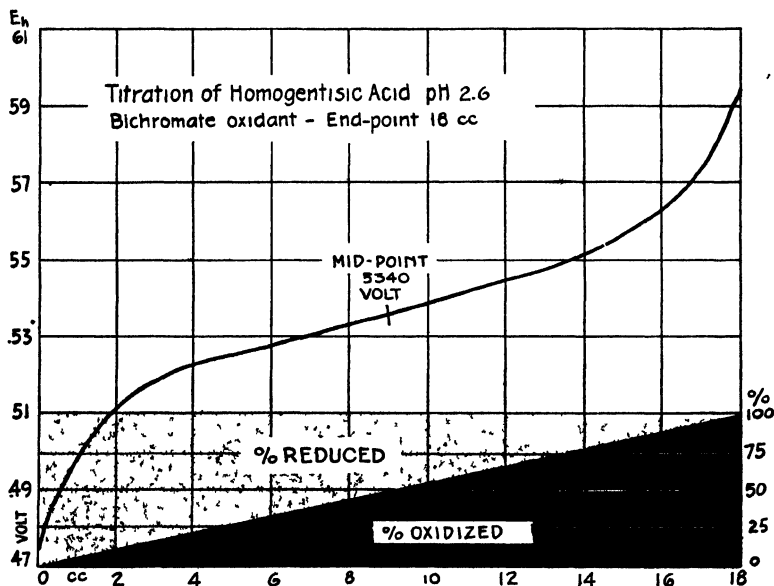


CHART 1. Oxidative titration of homogentisic acid. No precautions taken to exclude air.

lower pH than the other 2 from their respective hydroxyl linkages. The pH of a 0.02 N solution of homogentisic acid was 3.001.

Hence, since  $(H)^+ = \sqrt{AK_{r1}}$  where  $A$  is the total acid concentration,  $K_{r1} = 5.02 \times 10^{-5}$  and  $pK_{r1} = 4.28$  where  $K_{r1}$  is the first dissociation constant of homogentisic acid. Similarly, a quick determination of the pH of 0.02 benzoquinoneacetic acid gave pH 2.490 which corresponds to  $K_o = 5.05 \times 10^{-4}$  and  $pK_o = 3.28$ .

For the oxidized phase we must use  $(H)^+ = \sqrt{AK_o} - (K_o/2)$ .

( $K^2/4$  can be neglected in comparison with  $AK_0$ .) Half titration of the reduced phase gave a value similar to the above, but this method could not be applied to the oxidized phase. The first

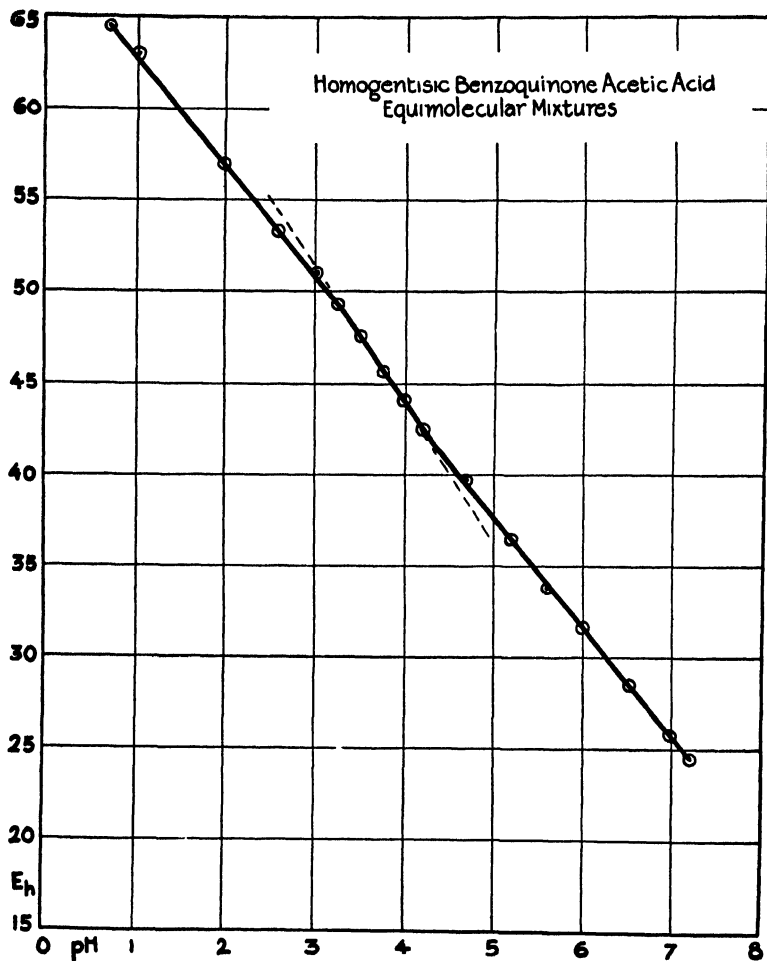


CHART 2. Relation of  $E_h$  to pH.  $pK_0$  and  $pK_1$  are defined by the intersection of the broken and solid lines.

pH determination gave a steady potential, but the addition of the least bit of NaOH resulted in the instant formation of highly colored products and unsteady potentials. Blix (7) reports 3.50

for  $pK_o$  and 4.14 for  $pK_r$ . His measurements were made at a temperature of  $20^\circ$ , while ours were made at  $25^\circ$ . An ingenious technique for the determination of such compounds has been developed by Ball and Chen (2) and Ball and Clark (8). Their potential ranges are higher than those reported by Blix and ourselves and they find an increase in potential by introduction of a carboxyl group into the molecule. La Mer and Baker (9) and Biilmann (10) found that the introduction of alkyl groups lowers

TABLE I

*Oxidation of Homogentisic Acid with Potassium Dichromate*

20 mg. of homogentisic acid in 75 cc. of buffer (NaCl + HCl) with potassium dichromate at same pH. End-point 18 cc. Temperature  $25^\circ$ . pH 1.984. No precautions taken to exclude air.

Oxidant		$\frac{RT}{2F} \log \frac{(S_o)}{(S_r)}$	$E_h$ observed	$E'_o$ calculated	Deviation
cc	per cent				
2	5 5	0 370	0 5328	0 5698	-0 0002
3	11 1	0 272	0 5429	0.5701	+0 0001
4	16 7	0 209	0 5493	0 5702	+0 0002
6	22 2	0 163	0 5544	0.5700	0 0000
7	27 8	0 124	0.5584	0.5708	+0.0008
8	44 4	0 029	0 5667	0 5696	-0 0004
9	49 9	0 000	0 5700	0 5700	0 0000
10	55 5	-0 029	0 5733	0 5703	+0 0003
11	61 0	-0 058	0 5755	0 5696	-0 0004
12	66 6	-0 090	0 5787	0 5698	-0 0002
14	77 7	-0 163	0 5862	0 5699	-0 0001
16	88 8	-0 270	0 5971	0 5701	+0 0001
17	94 4	-0 368	0 6070	0 5702	+0 0002

the potential. Hence some of the difference between the potentials of gentisinic acid and homogentisinic acid may be explained on the basis of the carboxyl group merely substituting in the side chain instead of in the ring. La Mer and Baker found an increase on introduction of halogen, to which the carboxyl group may be likened. However, the normal potential of dihydroxyphenylalanine of 0.797 in comparison with 0.69 of homogentisic acid remains to be explained. The difference in the dissociation constants of the oxidized and reduced phases should be apparent from Chart 2.



Applying the method of calculation outlined by Clark *et al.* (6) to this special case, we have according to the law of mass action

$$(1) \quad K_o = \frac{(\overset{+}{\text{H}})(\text{Ox}^-)}{(\text{HOx})}$$

$$(2) \quad K_{r_1} = \frac{(\overset{+}{\text{H}})(\text{RH}_2^-)}{(\text{RH}_3)}$$

$$(3) \quad K_{r_2} = \frac{(\overset{+}{\text{H}})(\text{RH}^-)}{(\text{RH}_2^-)}$$

$$(4) \quad K_{r_3} = \frac{(\overset{+}{\text{H}})(\text{R}^{\equiv})}{(\text{RH}^-)}$$

Let  $S_r$  = total reductant,  $S_o$  = total oxidant.

$$(5) \quad S_r = \text{RH}_3 + \text{RH}_2^- + \text{RH}^- + \text{R}^{\equiv} \quad (6) \quad S_o = \text{HOx} + \text{Ox}^-$$

$$(7) \quad \text{Ox}^- = \frac{K_o S_o}{(\overset{+}{\text{H}}) + K_o}$$

Eliminating  $\text{RH}_3$ ,  $\text{RH}_2^-$ , and  $\text{RH}^-$  from Equations 2, 3, 4, and 5 we obtain

$$(8) \quad \text{R}^{\equiv} = \frac{S_r K_{r_1} K_{r_2} K_{r_3}}{K_{r_1} K_{r_2} K_{r_3} + K_{r_1} K_{r_2} (\overset{+}{\text{H}}) + K_{r_1} (\overset{+}{\text{H}})^2 + (\overset{+}{\text{H}})^3}$$

When any pair which differs by 2 electrons is taken as the fundamental oxidation-reduction equation, we have

$$(9) \quad E_h = C - \frac{RT}{2F} \ln \frac{\text{R}^{\equiv}}{\text{Ox}^-}$$

If Equations 7 and 8 are substituted in Equation 9, there results

$$E_h = E_o - \frac{RT}{2F} \ln \frac{S_r}{S_o} + \frac{RT}{2F} \ln \frac{K_o}{K_{r_1} K_{r_2} K_{r_3}} + \frac{RT}{2F} \ln \frac{K_{r_1} K_{r_2} K_{r_3} + K_{r_1} K_{r_2} (\overset{+}{\text{H}}) + K_{r_1} (\overset{+}{\text{H}})^2 + (\overset{+}{\text{H}})^3}{(\overset{+}{\text{H}}) + K_o}$$

In all our considerations  $K_{r_3}$  and  $K_{r_2}$  may be neglected with respect to the other variables. At a fixed ratio of oxidant to reductant, here equimolecular quantities of each, with change in pH only the last term varies and we have

$$E_h = E_o^* + \frac{RT}{2F} \ln \frac{K_{r_1} (\overset{+}{\text{H}})^2 + (\overset{+}{\text{H}})^3}{(\overset{+}{\text{H}}) + K_o}$$

where  $E_o^*$  includes all constant terms.

Our  $E_h$ /pH curve passes through three regions in the range of pH measured and the variable term can be evaluated from the preceding equation.

Region A.  $(H)^+ > K_o > K_{r_1} > K_{r_2} > K_{r_3}$ . Variable term =  $0.059 \log (H)^+$

" B.  $K_o > (H)^+ > K_{r_1} > K_{r_2} > K_{r_3}$ . Variable term larger than  $0.059 \log (H)^+$ , increasing to a maximum, and then becoming smaller. Measurements are not accurate enough to show these changes on the curve

Region C.  $K_o > K_{r_1} > (H)^+ > K_{r_2} > K_{r_3}$ . Variable term =  $0.059 \log (H)^+$

It can be seen from Chart 2 that the dissociation constants are sufficiently far apart from each other to give the curve a bayonet

TABLE II  
*Relation of  $E_h$  to pH*

pH	$E_h$ Equimolecular mixtures	pH	$E_h$ Equimolecular mixtures
0 697	0 6465	4 007	0 4447
1 002	0 6291	4 225	0 4267
1 984	0 5701	4 801	0 3897
2 611	0 5341	5 207	0 3658
3 014	0 5104	5 618	0 3418
3 274	0 4941	6 004	0 3189
3 508	0 4771	6 534	0 2876
3 781	0 4582	6 994	0 2606
		7 201	0 2484

form. This system would have a potential of 0.265 at body pH and an rH of 22.5. From a biological point of view this is extremely positive and must cause changes in any other oxidation-reduction system of lower rH in its vicinity. This is in fact one of the highest systems to be actually shown as present in the body. Quinone itself is still higher and all the other similar compounds formed in the intermediate metabolism of the cyclic rings of protein are within this range. Another substance, adrenalin, has been shown by Ball and Clark (8) to have an rH of 27 ( $E'_0$  0.5395, pH 4.40) which also cannot be without biological significance.

The classic work of Kuester (11) showed that hemoglobin behaves as a ferrous salt (as also does oxyhemoglobin), while methemoglobin is a ferric salt. Conant (1) has shown that hemoglobin-methemoglobin is a reversible oxidation-reduction system whose potential at body pH is approximately +0.12 volt. The potential of the hydroxylated phenol systems at this pH is over 0.265 volt. Hence the circulation of these polyhydroxic phenols must cause a shift in the hemoglobin-methemoglobin system in favor of the formation of methemoglobin. The hemoglobin can cope with the normal amount circulating as a result of protein metabolism, but the added amount may shift the equilibrium, and *on this basis rests the formation of methemoglobin characteristic of poisoning by aniline derivatives*. Van Slyke and Vollmund (12) have shown that the action of aniline on methemoglobin *in vitro* showed a latent period at the beginning, no methemoglobin being formed for many hours. After methemoglobin formation began, it proceeded slowly and several mols of aniline per mol of hemoglobin were required to complete it. This behavior, they state, accords with the probability that a product of aniline rather than the aniline itself causes the methemoglobin formation. Thus the action of the body is necessary to secure the proper degree of oxidation of the compound to raise it above the hemoglobin-methemoglobin oxidation-reduction system potential. This is an extremely simple example of the familiar action of certain chemotherapeutic agents which cannot act in the test-tube but are potent in the body. A study of some of them, salvarsan, etc., shows that they are also potential oxidation-reduction systems.

We know that there must be a certain minimum of methemoglobin present to be measured spectroscopically and we can assume a small quantity as physiological. Various authors give the minimum as 2.5 to 5 per cent as the minimum apparent on spectroscopic examination. To hemoglobin, in addition to its many other regulatory functions in maintaining body equilibrium, must be added *the ability to poise correctly oxidation-reduction systems by virtue of its middle position in the biological oxidation-reduction scale*. We know that, if we add  $dA$  equivalents of a stronger oxidizing agent to a system in equilibrium where  $E = RT/nF \ln a/(S - a)$  ( $S$  is the total concentration of the reversible system and  $a$  the concentration of the oxidized form), the potential will

rise  $dE$ ,  $dE/da = RT/nF \cdot S/a(S - a)$ . The poising power  $\pi^* = nF/RT \cdot a \cdot (S - a)/S$ .  $a$  can vary only between 0 and  $S$ ,  $\delta\pi/\delta S = nF/RT \cdot a^2/S^2$ .

For finite values  $\pi$  has neither a maximum nor minimum, but the poising effect becomes greater the higher the concentration of the oxidation-reduction system. In other words, when a reversible system of middle position, such as Hb-MHb, and another of higher position are brought into contact, one or the other will predominate according to the molecular concentration. If the Hb-MHb is much more concentrated in comparison with the Q-QH, then it will completely predominate, which is the usual physiological condition. However, another factor comes into play. The molecular weight of hemoglobin, according to the latest and best available methods, assuming 4 iron atoms to the molecule, is 67,000:68,000. Since the quinones are approximately 100, small quantities of the quinones can exert enormous effects on the hemoglobin system. Theoretically 1 gm. of quinone (20 mg. per 100 cc. of blood) can convert the entire hemoglobin of the blood into methemoglobin and 1 mg. would be spectroscopically apparent. Hence, the introduction of hydroxylated phenols into the body at a concentration greater than the physiological maximum should cause the formation of methemoglobin. If we differentiate the poising effect by  $a$ , we find  $\delta\pi/\delta a = nF/RT ((S - a)/S - a/S)$ . At the maximum  $d\pi/da = 0$  and  $a = S/2$ . The second derivative shows this to be a maximum. Thus Docou (13), in an elaborate study of the effects of injection of acetanilide, resorcinol, and similar products into dogs, reports the formation of approximately 50 per cent methemoglobin.

Another aspect of this problem must be considered; namely, how is this equilibrium affected by the presence of oxygen? Passing  $O_2$  or CO into a mixture of hemoglobin and methemoglobin has been found by Conant to increase the oxidation-reduction potential of the mixture. He gives  $\pi$  (observed) =  $\pi_n + 0.059 \log (MHb)/(Hb)$ . If the term (Hb) is greatly diminished by combination of hemoglobin with the gas to form oxyhemoglobin or carboxyhemoglobin, and (MHb) stays the same, the potential will rise. Thus the potential at body pH of the system in the

\* This is not equal to Clark's "poising index," since it is dependent on absolute concentrations.

absence of oxygen is about +0.12 volt compared to the hydrogen electrode, while in the presence of  $O_2$  it rises to 0.2 volt. Thus the oxyhemoglobin acts as a bulwark in opposing the action of the highly positive oxidation-reduction systems and shows the reason for the comparative rarity of methemoglobinemia.

A peculiar form of methemoglobin formation first differentiated by Stokvis (14) as a clinical entity, "enterogenous cyanosis methemoglobinemia," leads to periodic violent intestinal attacks followed by extreme cyanosis with demonstrable high methemoglobin content of the blood. The urine contains a substance which on being exposed to air turns bright red and is able to convert hemoglobin to methemoglobin *in vitro*. The condition has been referred to absorption into the blood stream of some toxic substances formed in the intestine by putrefactive change. In the intestinal contents there have been reported phenol-*p*-cresol, *p*-hydroxyphenylpropionic acid, etc., all of which can be converted into polyhydroxy phenols by the body and hence are potential methemoglobin formers.

An interesting aspect of the equilibrium between hemoglobin-methemoglobin and other oxidation-reduction systems is given in the paper of Michaelis and Salomon (15). The change of oxyhemoglobin by an oxidizing agent into methemoglobin results in the simultaneous reduction of the oxidant and the liberation of a certain quantity of oxygen from the oxyhemoglobin. 1 mol of oxygen is liberated for every mol of hemoglobin formed. This is the case with ferricyanide and quinone. If, however, the reduction product is autoxidizable, then some of the oxygen is used up in reoxidizing the dyestuff. Hence, when an organic dye is used, three-quarters of the oxygen should be produced which is formed by ferricyanide, if all the hemoglobin is converted into methemoglobin. At least, this happens when an excess of dye is used and every molecule acts only once as an oxidant of the hemoglobin. However, in making the list of the amounts liberated by the various commonly used oxidation-reduction systems, they find that less than three-quarters is liberated (at pH 6) except in the case of chlorophenolindophenol ( $E'_0 = +0.295$ ), phenolindophenol (+0.28). Methylene blue (+0.047) gave only half this quantity "in nicht gut reproduzierbarer Weise," indigo sulfonate (-0.006) only a fifth, safranine (-0.2), and rosinduline practi-

cally none at all. This is to be expected because chlorophenolindophenol, phenolindophenol, and quinone are the only systems whose  $E'_0$  exceeds that of the hemoglobin-methemoglobin system (approximately +0.09).

The case of methylene blue is exceedingly interesting. As can be seen from the above, it is practically in the potential range of the hemoglobin-methemoglobin system, and if we take Conant's measurements as of the precision of other more easily investigated systems, a little lower. It can be easily calculated how much methemoglobin can be formed. Methylene blue as commonly used has a minimum amount of leuco product. Let us assume that at equilibrium between the two systems, which start at equimolecular concentrations of hemoglobin and methylene blue,  $x$  equivalents of methemoglobin are formed. We have

$$\begin{aligned} S_{Mb_f} &= S_{Mb_i} - x & S_{MHb_f} &= S_{MHb_i} + x \\ S_{Lmb_f} &= S_{Lmb_i} + x & S_{Hb_f} &= S_{Hb_i} - x \end{aligned}$$

where MHb is methemoglobin, Hb hemoglobin, Lmb leucomethylene blue, and Mb methylene blue, and  $i$  and  $f$  are the initial and final concentrations respectively.

At constant pH the systems are separately defined by

$$E_m = E'_m + 0.03 \log \frac{(S_{Mb})_i}{(S_{Lmb})_i} \quad E_b = E'_b + 0.06 \log \frac{(S_{MHb})_i}{(S_{Hb})_i}$$

The systems react to a common potential  $E_m = E_b$ . Hence

$$E_b - E'_m = 0.03 \log \frac{(S_{Lmb})_f (S_{MHb})_f^2}{(S_{Hb})_f^2 (S_{Mb})_f} = 0.03 \log \frac{x^3}{((S_{Hb})_i - x)^2 ((S_{Mb})_i - x)}$$

where  $(S_{Lmb})_i = 0$  and  $(S_{MHb})_i = 0$ . Using the values given by Conant for  $E'_b$  and the equivalent value at a definite pH of methylene blue, we have  $x = 0.106$  concentration of hemoglobin. Hence approximately 10 per cent would be converted into methemoglobin, and the reaction would stop. Another factor enters here; when air is admitted, the leucomethylene blue is instantly reoxidized to methylene blue and the reaction goes on continu-

ously. Thus methylene blue can be regarded as a positive system only in the presence of air, it is a facultative positive system in contrast to the obligate systems previously discussed. The systems of this range, the facultative positive, are the systems which play the greatest rôle in catalyzing respiration. In contrast to the obligate systems, the strongly positive ones spoken of previously, they are dependent on the concentration of the reacting systems to a greater extent. Expressed otherwise, the important factor is not the absolute difference in the characteristic potential ( $E'_m$  and  $E'_b$  in the last few equations), but the spread between the systems achieved by having the oxidant of the one system differ so extremely in its proportions from its reductant, and the reductant of the other system differ so much from its own oxidant. These must be extremely unstable systems and in a state of constant flux, because we know that these are the regions of great instability, as stability is really only achieved where the oxidant and reductant tend to approach each other in relative concentration. This is admirably adapted to respiratory needs. Warburg (16) found that the methemoglobin formed by the action of the very positive systems differs very much from that formed by methylene blue in its ability to catalyze oxidation, and that a much higher concentration of the former was required to attain the same velocity of oxidation as with the latter.

Some light is thrown on the old question as to why the pigment in ochronosis is so strictly localized in the cartilages and scleræ. Virchow states in the original description, "I will name this condition ochronosis. It is strictly localized in those parts that are without nerves and blood vessels." These are the regions where there can be no poisoning effect of hemoglobin. Hence the quinone from the homogentisic acid can be formed and at the pH of the body fluids instantly form the colored product characteristic of ochronosis. "The natural question is whether any physiological analogy can be found. It seems to me it can be found in the hair, which is dependent on the same condition."

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## DERIVATIVES OF GLUCURONIC ACID

### III. THE SYNTHESIS OF DIACETYLCHLOROGLUCURON

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The fact that glucuronic acid has been found as a constituent of certain of the specific polysaccharides derived from encapsulated pathogenic microorganisms (1) has lead us to a study of the chemistry of this interesting acid and its lactone glucuron. The type-specific bacterial polysaccharides are of great immunological importance (2), for it is these substances, as they occur in the intact bacterial cell, which orient the production of specific antibodies in immunized animals, and which ultimately render the animal immune to infection with virulent organisms of the homologous specific type. When these bacterial carbohydrates are hydrolyzed with mineral acids, in some instances sugar acids are found among the products of hydrolysis (3). These sugar acids, a new type first described in this laboratory, were shown to be glucose glucuronides and were termed aldobionic acids. Not only are the bacterial polysaccharides of great immunological importance, but their partial hydrolytic products and even the aldobionic acids themselves are now known to be immunologically specific (4).

In the previous communications (5) of this series the preparation of glucuronic acid from glucuron and the preparation and separation of  $\alpha$ - and  $\beta$ -triacetylglucuron were described. The present communication presents a method for the synthesis of diacetylchloroglucuron. The successful preparation of this latter derivative of glucuronic acid should make possible the synthesis of glycosides of glucuronic acid, and the synthesis of the aldobionic acids as well.

With reference to the preparation of acetohalogen derivatives of glucuron, we have already pointed out that in 1905 Neuberg and

Neimann (6) reported the preparation of crystalline acetobromoglucuron, in yields of 13 per cent, by the action of acetyl bromide on glucuron. However, we have thus far not succeeded in confirming the results of their experiments, nor have we succeeded in preparing this derivative in crystalline form by employing methods more recently devised for the preparation of acetohalogen sugars (7). We attribute our failure to the fact that acetobromoglucuron appears to be an unstable substance, and that products of decomposition apparently interfere with the successful isolation of a crystalline end-product. For this reason we turned to the preparation of a chlorine derivative, and we found that diacetylchloroglucuron could be easily prepared by the action of hydrogen chloride and acetyl chloride on triacetylglucuron. It was only after many attempts, however, that this derivative was first secured in crystalline form, but subsequently there has been no difficulty in crystallizing later preparations.

Diacetylchloroglucuron is a very stable derivative; it may be kept for many weeks in a desiccator without signs of decomposition. The substance is quite soluble in chloroform and acetone, but dissolves with difficulty in anhydrous ether. When an ethereal solution of acetochloroglucuron is treated with moist silver carbonate, the chlorine atom is replaced by hydroxyl to yield diacetylglucuron, just as acetobromoglucose yields tetraacetylglucose under similar conditions. Diacetylglucuron may be recrystallized from alcohol, but this operation is accompanied by an appreciable loss in material. This is due to the fact that the diacetyl derivative mutarotates rapidly in solution to yield a more levorotatory substance, probably an isomer, which we have not obtained in crystalline form. In addition to being soluble in alcohol, the diacetyl derivative may be dissolved in hot water, but it will not crystallize from this solvent. The compound apparently decomposes during this operation, because the solution, at first neutral, rapidly becomes acid. This may be due either to the opening of the lactone ring, to the splitting off of acetyl groups, or to both reactions occurring simultaneously. In many respects this compound behaves similarly to the tetraacetylglucose described by Fischer and Delbrück (8).

The fact that diacetylchloroglucuron yields diacetylglucuron seems to prove beyond doubt that the chlorine atom of the former

compound is attached to carbon atom (1) in that derivative. In regard to the structure of diacetylchloroglucuron, it is probably 1-chloro-2,4-diacetylglucuron, though there is no evidence to support this assumption beyond the fact that Pryde and Williams (9) believe that glucuron itself is a 1,5 lactal, 3,6 lactone of glucuronic acid. It is not known, furthermore, whether diacetylchloroglucuron is an  $\alpha$  or a  $\beta$  derivative.

In conclusion it is hoped that the successful preparation of diacetylchloroglucuron will now make possible the preparation of glycosides of glucuron and of glucuronic acid as well as the synthesis of aldobionic acids. Further work on these problems, and on the preparation of an acetone derivative of glucuron is now in progress.

#### EXPERIMENTAL

*Diacetylchloroglucuron*—10 gm. of a mixture of  $\alpha$ - and  $\beta$ -triacetylglucuron were dissolved in 50 cc. of redistilled acetyl chloride in a pressure bottle. The solution was cooled with solid carbon dioxide and then saturated with dry hydrogen chloride. The bottle was sealed and allowed to stand for 2 days at room temperature. After again cooling with carbon dioxide, the bottle was opened, the contents placed in a distilling flask, and the acetyl chloride removed *in vacuo*. The oily residue was dissolved in 100 cc. of chloroform, the solution cooled in ice, and washed successively with small portions of cold 0.1 N sodium bicarbonate solution, and finally with ice water. The chloroform solution was now dried with anhydrous calcium chloride, then filtered, and the chloroform removed *in vacuo*. The residue in the flask was next triturated with anhydrous ether, seeded with a small crystal of diacetylchloroglucuron,<sup>1</sup> and the flask placed in the ice chamber overnight. Crystals of diacetylchloroglucuron were filtered and washed well with anhydrous ether. 7.6 gm. of material were recovered, a yield of 83 per cent of the theoretical. The compound was recrystallized by dissolving in 15 cc. of chloroform, followed by the addition of 40 cc. of anhydrous ether. 7.4 gm.

<sup>1</sup> The first crystals of diacetylchloroglucuron were obtained by dissolving the oily residue, secured at this point, in warm anhydrous ether, and allowing the solution to stand for several weeks in the ice box. The oil which separated from the ethereal solution ultimately crystallized.

of crystalline substance were thus recovered. The compound melted sharply at 107.5–108.5° (corrected) preceded by a slight softening at 103° (corrected). The specific optical rotation was

$$[\alpha]_D^{25} = \frac{+2.40 \times 100}{2 \times 1.257} = +95.5^\circ \text{ (in chloroform)}$$

A second recrystallization changed neither the melting point nor the specific rotation of the derivative. The substance had the following composition.

7.525 mg. substance:	3.800 mg. AgCl
4.050 " " "	: 6.350 " CO <sub>2</sub> , 1.470 mg. H <sub>2</sub> O
C <sub>10</sub> H <sub>11</sub> O <sub>7</sub> Cl.	Calculated. Cl 12 73, C 43 09, H 3.99
	Found. " 12 49, " 42 76, " 4.06

*Diacetylglucuron*—5.2 gm. (1.5 mols) of freshly prepared dry silver carbonate were added to a solution of 7 gm. of diacetylchloroglucuron in 600 cc. of anhydrous ether. 0.2 cc. of water was added and the solution was shaken at room temperature. At first the evolution of carbon from the mixture was rapid, but in order to bring the reaction to completion, the solution was shaken overnight. A test sample now showed no chlorine to be present in the ethereal solution. The ether was removed by filtration and the precipitate of silver salts was extracted with hot alcohol, from which a fairly large amount of material crystallized on cooling. The ether was concentrated *in vacuo* to a volume of 30 cc. and from the solution a small amount of crystalline substance was also recovered. The products from the alcoholic and ether solutions were found to be identical so they were filtered together, and washed with a small amount of cold alcohol and ether. 5.3 gm. of diacetylglucuron were thus recovered, a yield of 80 per cent of the theoretical.

<i>Analysis</i> —4.870 mg. substance:	8 220 mg. CO <sub>2</sub> , 2.090 mg. H <sub>2</sub> O
C <sub>10</sub> H <sub>12</sub> O <sub>8</sub> .	Calculated. C 46.14, H 4 65
	Found. " 46 03, " 4 80
Acetyl determination (Pregl and Soltz's (10) method)	
17.732 mg. substance used	9.61 cc. N/70 NaOH
C <sub>10</sub> H <sub>12</sub> O <sub>8</sub> .	Calculated. CH <sub>3</sub> CO 33 06
	Found. " 33.29

The compound was recrystallized rapidly from ethyl alcohol, yielding about 4.5 gm. of a product which melted at 130–131°

(corrected). Subsequent crystallizations did not raise this melting point. A weighed sample of the crystalline product was dissolved in a given volume of absolute methyl alcohol, and the optical rotation was observed. Within the observation period of 25 minutes it was found that the solution mutarotated very rapidly.

*Optical Rotation*—0.5610 gm. of diacetylglucuron per 100 cc. of solution gave the following rotations when observed in a 2 dm. tube.

Time of observation after complete solu- tion of material min.	Observed rotation degrees
2	+1 60
3	+1 45
4	+1 09
6	+1 06
9	+1 02
10	+0 97
25	+0 85

#### SUMMARY

1. A method for the preparation of diacetylchloroglucuron has been given.

2. The conversion of diacetylchloroglucuron to diacetylglucuron has been described.

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## QUANTITATIVE STUDIES OF THE COMPOSITION OF GLOMERULAR URINE\*

### VII. MANIPULATIVE TECHNIQUE OF CAPILLARY TUBE COLORIMETRY†

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For some years past, in this and in two other laboratories, studies of fundamental problems of renal function have been made by collecting and analyzing fluid taken from single units of the living kidney. Thus far, the work has been confined almost wholly to glomerular puncture and analysis of glomerular urine from frogs and *Necturi*. The method of glomerular puncture, originated by Wearn and Richards (1), has gradually been perfected so that now it is not difficult to make collections of glomerular urine without recognizable injury to the glomerulus, against positive pressures, and without contamination from surface or

\* Previous studies in this series have not been numbered; they are to be found in this *Journal*, **66**, 247 (1925); **87**, 467, 479, 499, 523 (1930); **91**, 593 (1931).

† The development of this technique in relation to the estimation of uric acid was begun in the Laboratory of Pharmacology of the University of Pennsylvania in the autumn of 1930 and continued in the Laboratory of Physiology of the Harvard School of Public Health in the spring of 1931 during the occupancy by Richards of a visiting professorship and by Bordley of a research fellowship in that institution. Grateful acknowledgements are made to the authorities of Harvard University and to Professor Cecil K. Drinker for the facilities of his laboratory. Development of the technique in relation to the estimation of glucose was undertaken by Walker in Philadelphia in the spring of 1931. The expenses of this investigation have been defrayed in part from a grant by the Commonwealth Fund.

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interstitial fluid or from tubule contents. The volumes of glomerular fluid obtainable in a single experiment are so small, however, that methods sufficiently refined for their quantitative analysis are few. Wearn and Richards (2) and Freeman, Livingston, and Richards (3) determined chlorides by an adaptation of T. W. Richards' nephelometric method. Ekehorn (4), working in Oxford, devised a microtitration method for the same purpose. Schmitt and White (5) made a quantitative study of phosphates in glomerular urine, bladder urine, and plasma by a capillary adaptation of the method of Benedict and Theis. White (6) and later Walker (7) compared total molecular concentrations of glomerular urine and plasma by utilizing Barger's capillary method for the estimation of molecular weights. Bayliss (8) developed a technique for determining electrical conductivity of minute amounts of fluid and applied it to this problem. Walker (9) determined urea by an adaptation of the hypobromite method to volumes as small as 0.1 c.mm. Richards and Walker (10) made a quantitative study of the glomerular elimination of phenol red and indigo carmine by utilizing the fact that approximately accurate quantitative colorimetric comparisons can be made of minute amounts of fluids contained in capillary tubes. In so far as we are aware, these are the only analytical methods which have been found applicable to the problem.

The results of these efforts have added support to the doctrine of glomerular filtration, although the evidence is not unanimous. Unequivocally in support are the comparisons of electrical conductivity, urea concentration, and dye concentration of glomerular urine and plasma, the chloride estimations by Ekehorn, and Walker's series of estimations of total molecular concentration. The results of chloride determination by Wearn and Richards were contrary to the filtration theory; those of Freeman, Livingston, and Richards were ambiguous. White's estimations of total molecular concentrations and of phosphates are in opposition to the doctrine of filtration.<sup>1</sup> These statements represent the status of the problem of glomerular function in so far as it had been studied by these methods at the time of the beginning of the work now to be reported.

<sup>1</sup> White's most recent work has led him to reverse this conclusion.

For two reasons it seemed unquestionably important to extend the list of quantitative chemical methods which should be reliably applicable to the analysis of the minute amounts of fluid which concern us in this general problem: to establish more firmly the truth or falsity of belief in filtration as the sole glomerular process, and to accumulate added resources of method for an attack upon problems of the function of the tubule similar in design to that which has been applied to the function of the glomerulus. The work which is to be described in the papers which immediately follow this derived from both of these considerations. From experience with colorimetry in capillary tubes, gained in the study of the elimination of dyes by the kidney (9, 11), we acquired the conviction that this method was susceptible of wider and more important applications. The colors which are developed when current methods for the estimation of glucose and phosphates are applied to solutions as concentrated in these constituents as is normal blood plasma are so intense that they are conspicuously apparent when the reaction mixture is contained in a capillary tube of less than 0.5 mm. inner diameter. This statement is not literally true of uric acid and creatinine, or the substances in blood responsible for Jaffe's reaction, but, by injection of small amounts, the plasma uric acid or creatinine can be increased to the level at which it becomes true.

It therefore appeared that to make current methods for the quantitative determination of a number of important constituents of blood and urine applicable to such minute quantities of fluid as are obtainable from single renal structures, only a manipulative technique is required, which should enable the analyst to mix, in minute containers, the unknown and standard solutions with the appropriate reagents in correct proportions, to conduct the reactions under standard and uniform conditions, and to make accurate comparisons of the resulting colors. Such a technique has been developed and applied to the four substances mentioned.<sup>2</sup> The details and results of these applications will be described in Papers VIII to XI of this series. In this paper we shall describe the manipulative technique which is common to all.

The problem, briefly stated, is this: to introduce the minute

<sup>2</sup> Work is in progress in this laboratory on similar determinations of chloride, sulfate, and iron.

amount of fluid to be analyzed into a capillary tube without evaporation or contamination, to dilute it quantitatively with water if necessary, to introduce into the same capillary in quantitatively accurate proportions and without mixing the one or more reagents required for production of color, to effect mixture of the fluids in the capillary tube at a given moment, and to compare the resulting color with those developed in standard solutions treated simultaneously in identical or equivalent fashion.

### *Apparatus*

*Capillary Tubes*—Two sizes of capillary tubing have proved most useful: the smaller is 0.5 mm. outside, 0.35 mm. inside diameter; the larger is 0.8 mm. outside diameter, 0.6 to 0.7 mm. inside. The smaller tubes will frequently be referred to as “mixing capillaries” for it is in these that measurements and mixture of solution and reagent are effected and color developed. The larger tubing is used for blood collections, for plasma protein precipitations, and to make pipettes. It is essential that the smaller tubing used in any one experiment shall be of uniform inner diameter. Only a small proportion of capillary tubing drawn by the hands of even a skilled glass worker meets this requirement; hence we have obtained tubing mechanically drawn, which is more uniformly perfect.<sup>3</sup> It is made in 2 foot lengths from thoroughly cleaned Pyrex glass tubing and is stored in dust-proof tubes.

*Pipettes*—For all transfers of fluids described in this paper pipettes, made from the larger capillary tubing, are used. They are drawn out to a slender tip, about 50 $\mu$  outside diameter; 10 cm. is a convenient length. A supply of these, together with rubber tubing and mouthpiece to fit, should be prepared before beginning a series of determinations.

*Microscope*—Transfers and measurements of fluids are made with the aid of a binocular microscope equipped with lenses to give about 15-fold magnification. The optical field should be about 1 cm. in diameter. Microscopic measurements are made either with a micrometer disc inserted into one of the oculars or

<sup>3</sup> From the International Resistance Company, 2006 Chestnut Street, Philadelphia.

with a stage micrometer made by cementing the disc to the glass stage of the microscope. The scale is 10 mm. long, ruled in 0.1 mm. divisions. When in use, the glass stage, with the exception of the circle corresponding to the optical field, is covered with wet filter paper in order to lessen the chance of evaporation of fluids handled in the field.

*Water Manipulator*—To introduce separate, successive columns of fluid into one end of a capillary tube and to hold these stationary in the microscopic field for measurement of length, it is necessary to be able to apply gentle, controllable suction or pressure at the other end. A small syringe has been constructed with a piston, 3 mm. in diameter, which can be advanced or withdrawn by a fine micrometer screw. The tip of the syringe is connected by rubber tubing with a short glass or metal tube drawn out to a point somewhat smaller in outside diameter (about 0.2 mm.) than the inside diameter of the capillary tube into which it is to project. The syringe, rubber tube, and tip, mounted on a stand at the level of the microscope stage, are filled with colored water. Air bubbles must be excluded. When water is forced through the slender tip into a capillary tube fitting loosely over it, a water seal is effected tight enough to permit movement of water into or out of the capillary tube without loss. Such movements of water provide with great nicety the suction or pressure required to control the movements of columns of fluid introduced into the other end of the capillary.

*Small Centrifuge, Driven Either Electrically or by Hand*—If the latter is used, it is convenient to have it equipped with a hematocrit head and to have a supply of glass tubes, sealed at one end, to fit this.

A piece of milk glass with unglazed surface, 35 cm.  $\times$  35 cm.  $\times$  4 mm., two desk lamps equipped with 100 watt bulbs, one suspended lamp with a 150 watt bulb and daylight glass screen complete the necessary equipment.

### *Procedures*

Assume that to be analyzed is a solution of a substance, which, like uric acid, requires for the development of color the separate addition of two reagents in prescribed proportions. Reagents, apparatus, and a series of standard solutions of appropriate range

are ready. A 10 to 12 cm. length<sup>4</sup> of the smaller capillary tubing is connected with the water manipulator in the manner described above. It is then fixed upon the stage of the microscope so that its axis parallels the micrometer scale and its open end is visible in the optical field near the edge of this which is furthest from the manipulator. Water is forced from the manipulator into the capillary tube until about half its length is filled. A small amount of the solution to be analyzed is drawn into the tip of a pipette;<sup>5</sup> this is brought immediately into the field of the microscope and inserted into the open end of the capillary. Gentle pressure with the breath through the mouthpiece of the pipette starts flow of solution into the capillary. Simultaneous rotation of the milled head of the micrometer screw of the water manipulator draws the fluid inward so that the outer meniscus is never stationary at the extreme end of the tube. By this combination of pressure and suction it is easy, with practice, to introduce the solution as a column of predetermined length, exactly measured by reference to the graduations of the micrometer scale which are visible through the capillary tube. After the measurement has been verified, this column is drawn in further from the end of the tube until its distal meniscus is near the center of the field. In the same manner, from a second pipette, a measured column of the first reagent is introduced and its measurement verified. The two columns are then drawn further in and the second reagent in a measured column is introduced. The three columns are then drawn in until the distal meniscus of the third is about a centimeter from the end of the tube. The part of the tube, some 3 to 4 cm. in length, which contains the three fluid columns is quickly broken off, its two ends sealed in a minute gas flame, and laid aside in a horizontal position. The complete preparation of a capillary in this fashion requires less than 2 minutes.

It is unnecessary to measure the *volumes* of the fluid columns; their *lengths* are the only accurate measurements necessary and

<sup>4</sup> The edge of a carborundum block is a better instrument for cutting capillary tubes than either a file or a glass knife. It is convenient to have it supported on a stand within easy reach of the microscope.

<sup>5</sup> When glomerular urine is analyzed it may be introduced directly from the collecting pipette into the mixing capillary.

these are chosen to be proportional to the volumes of fluids which are specified in the corresponding macromethod.

The following precautions are important. Measurements of the length of each of the columns should be made from the same zero point on the micrometer scale. The distal end of the tube which is broken off must not include any of the tube which has been wet with water from the manipulator. When the ends of the tubes are sealed, care must be taken not to heat the column of fluid nearest the flame.

We now have a tube containing three measured columns of fluid, separated by bubbles of air which prevent mixing until means are used to make them mix. The order in which the reagents are introduced determines the order in which their mixture with the fluid to be analyzed subsequently occurs.

In exactly the manner above described, a series of tubes is prepared from standard solutions of the appropriate concentrations. Since the length of each tube is its only means of subsequent identification, each succeeding tube is made a little longer than its predecessor.

All of the tubes, arranged so that the column of reagent last introduced is uppermost, are placed in one of the tubes of the centrifuge. The centrifuge is whirled at high speed for a few seconds; the three columns of fluid in each tube are thrown together at the end. To insure thorough mixture, the tubes are removed from the centrifuge, reversed, replaced, and again centrifuged for a few seconds. This is repeated a second time. When heat is required for full development of color, the tubes, after removal from the centrifuge, are bound together and immersed in water of the required temperature for the required time. The fluids are then ready for color comparison.

*Color Comparisons*—For these it is essential that all of the colored solutions be contained in tubes of the same diameter. When a series consists of only five or six tubes, it is not difficult to choose a single length of tubing sufficiently uniform in bore to permit color comparisons to be made in the original mixing capillaries. When there are ten to fifteen tubes in a series, the uniformity of the several lengths of tubing required may not be such as to allow this. In this case an additional step is introduced into the procedure preliminary to the color comparisons. A single length

of the 0.35 mm. tubing is selected, about 30 cm. long, known from careful measurements to be uniform in bore throughout its entire length.<sup>6</sup> This is broken into pieces each about 2 cm. long. All the standard and unknown solutions in which color has been developed as above described are transferred to these pieces. To do this, the sealed ends of the tubes are broken, one end inserted into small rubber tubing held in the mouth, the other held in contact with one end of the piece into which transfer is to be made. Gentle pressure results in instant transfer of the fluid from one tube to the other; the ends of this are quickly sealed with plasticine. Two precautions are necessary: when the fluid is forced from one tube into the other, the two tubes should be held in the position of a wide angled V; if they are held in the same line, it is easy to blow the fluid through and out of the second tube. The plasticine must not come in contact with the colored fluid.

As soon as each tube is finally ready for color comparison, whether or not the step just described has been necessary, it is placed in a labeled space on the milk glass plate on which color comparisons are to be made. For blue colors this is illuminated by two desk lamps, the 100 watt bulbs of which are placed side by side about 6 inches above the plate. The use of two bulbs prevents shadows. For colors at the red end of the spectrum a 150 watt lamp provided with suitable color screens is suspended above the plate. When the standard tubes are placed one at a time beside the unknown, it is easy to select the lowest standard whose color is definitely more intense and the highest standard whose color is less intense than that of the unknown. More careful comparison must then be made to decide whether the color intensity of a standard tube is identical with that of the unknown, and if not, as is usually the case, to assign a value to the unknown, intermediate between the two nearest standards. In these finer discriminations it is important that the tubes be parallel and flat against the glass; it is helpful to cover them with white paper in which a rectangular window has been cut, the width of which is such that the visible columns are of the same length.

In the above description there is the implication that it is not permissible to make the color mixtures of the *standard* solutions in

\* Measurements of diameter were made with a Zeiss filar micrometer. Variations in diameter of 2 per cent are permissible.

the usual (macro) way in test-tubes and to use minute portions of these, taken into capillary tubes, as standards of comparison. It cannot be assumed that the intensity of color developed when minute quantities of fluid and reagent are mixed in a capillary tube is the same as when the same fluids are mixed in the same proportions in larger quantities in test-tubes. In two of the four determinations with which these papers deal (uric acid and phosphates) this assumption is not true. When it is true the procedure can be made less laborious by preparing the standard color mixtures in test-tubes and by taking samples of these for comparison into capillary tubes of the same caliber as that containing the unknown.

*Preliminary Tests and Dilutions*—The concentration of a substance which is to be determined by this technique must lie within a restricted range. Below a certain concentration the colors developed are too faint and above a certain concentration too intense to make accurate differentiation possible. Hence a preliminary test may be necessary.<sup>7</sup> If found to be more dilute than the lowest standard, it is not suitable for analysis by this method; if more concentrated than the highest standard it must be diluted.

A 2-fold dilution is best made in the mixing capillary. Half the usual volume of unknown is introduced and the column brought up to the required length by the direct addition of water. The reagents are then introduced in the manner described. For higher dilutions an extra step is necessary. The unknown solution and the required amount of water are introduced as separate columns into a small capillary and mixed by three centrifugations. The fluid is then drawn into a pipette the tip of which already contains a little oil; it is then ready for transfer to the mixing capillary.

The use of oil,<sup>8</sup> as indicated above, is a precaution against evaporation. Whenever, in these manipulations, it is necessary to allow a small amount of fluid to remain in a pipette for more than

<sup>7</sup> In our comparisons of glomerular urine and plasma, it has been practicable to make all preliminary tests on plasma because of the approximate identity of the two fluids with respect to concentrations of the substances under discussion.

<sup>8</sup> Through the courtesy of the Atlantic Refining Company, we have obtained a mineral oil of low viscosity, designated 250 T, which is well adapted to this purpose.



a few seconds, oil is taken into the pipette before and after drawing in the fluid. Thus neither surface is exposed to the air. When two or more portions of the fluid are to be taken from the pipette within a few seconds, the second column of oil is not necessary, provided the pipette in the interval is placed in a chamber (museum jar or inverted beaker), the walls of which are lined with wet filter paper. These precautions are unnecessary in the transfer of reagents or of fluid to be analyzed if the supply is relatively large. Pipettes are filled with these at the beginning of a series of preparations and kept in the moist chamber when not actually in use. (The creatinine reagent is an exception (12).) A little of the reagent is discharged onto a piece of filter paper immediately before inserting the tip into the mouth of the mixing capillary.

*Preparation of Protein-Free Plasma Filtrates in Capillary Tubes*—In previous work in this laboratory, blood, taken during the course of a glomerular fluid collection, has been drawn from the anterior abdominal vein. In the frog experiments reported in this group of papers, blood collections have been made directly from the ventricle of the heart by thrusting the fine, sharply pointed tip of a capillary pipette through the wall. The pipette is made from the larger capillary tubing. The apex of the ventricle is held by fine forceps, the points of which are guarded with cotton. The point of the pipette is thrust through the thickest part of the muscle at the apex. 0.02 cc. of blood (about 7 cm. in the pipette) yields an ample supply of plasma for several determinations. A few grains of dry sodium oxalate may be placed in the pipette before drawing the blood if oxalate does not interfere with the subsequent analysis. In any case, excess of oxalate must be avoided.

After the blood has been taken into the pipette, the larger end is sealed in a minute gas flame and the tube immediately centrifuged. If desired, measurement of percentage cell volume may be made at once by a rule. The tube is then cut a little above the juncture of cells and plasma, the plasma column allowed to flow by gravity back from the cut end, and both ends of the plasma tube sealed. Due precaution must be taken not to heat either blood or plasma when the ends of tubes containing them are being sealed.

Protein-free filtrates from plasma are prepared by tungstic acid precipitation (Folin-Wu) as follows: A large capillary tube (0.6 mm. inside diameter) is attached to the water manipulator and adjusted under the objective of the microscope. A column of 2/15 N  $\text{H}_2\text{SO}_4$ , 5.0 mm. long, is taken in from a reagent pipette and drawn back 5 mm. from the end. A column of plasma 4 mm. long is then taken in from another pipette and to it is added 10 per cent sodium tungstate to increase the length to 5 mm. After these two columns have been drawn further into the tube, they are made to oscillate back and forth a number of times by intermittent pressure with the water manipulator. This effects more thorough mixture of the sodium tungstate with the plasma. The distal part of the tube is then broken off and the ends sealed in the flame. That nearest the acid is sealed last and held in the flame long enough to produce a small bulb. The tube is placed in the centrifuge, bulb end down, and whirled. Mixture of the fluids and precipitation of proteins take place in the bulb. At least six times the tube is reversed in the centrifuge and whirled in order to make precipitation complete and to wash thoroughly each particle of precipitate with the fluid in which it is suspended. The final centrifugation carries the fluid and precipitate into the narrow end of the tube and is continued vigorously for several minutes. The volume of the clear supernatant fluid is then about two-thirds of the total. The tube is broken about 5 mm. above the surface of the fluid and the protein-free "filtrate" drawn off into a pipette. From the volume of plasma specified above enough fluid is obtained for several analyses.

A zone of haziness between clear fluid and precipitate indicates the presence of too much oxalate.

It will be noted that in this precipitation we use 1 part of 10 per cent sodium tungstate to 4 parts of plasma, whereas in the Folin-Wu procedure the ratio is 1:1. The low protein content of frog plasma is the reason for this divergence. The proportion of  $\text{H}_2\text{SO}_4$  added in the two procedures is the same. The final dilution of plasma in the Folin-Wu method is 1:10; in our adaptation, 1:2.5. For capillary tube colorimetry, dilution of plasma must be kept as low as possible. The dilution chosen was found to be the lowest consistent with completeness of separation and adequacy of volume of the protein-free fluid.

In the estimation of plasma phosphates, trichloroacetic acid is used as protein precipitant (Bell and Doisy). In our adaptation a column of plasma 4 cm. long, measured by a steel rule, is introduced into a piece of the larger capillary tubing. This is followed by a column of 90 per cent trichloroacetic acid (by weight) 1 mm. long, measured under the microscope. The tube is sealed and centrifuged with the plasma column down. It is then removed, immersed in hot water for a moment, and again centrifuged several times. The position of the tube in the centrifuge is reversed before each succeeding centrifugation. If then the supernatant fluid appears turbid, it is separated from the precipitate by cutting the tube, drawn into another pipette, the large end of this sealed, and the fluid subjected to a last vigorous centrifugation. The sediment is separated by cutting the tube, leaving in the pipette the clear, protein-free "filtrate" ready for transfer to a mixing capillary in which the color reaction is to be developed.

*Estimation of Protein in Minute Amounts of Fluid*—The precipitation reactions for protein as originally applied in examinations of glomerular urine (2) have been refined to a roughly quantitative method of estimation as follows: A measured column (1 to 2 mm.) of the fluid to be tested (diluted or undiluted glomerular urine) is introduced into a small capillary tube, the microscope and water manipulator as described above being used. An equal volume of 20 per cent trichloroacetic acid is added and thorough mixture effected by movement back and forth within the tube. Evaporation is prevented by a drop of water in the extreme distal end of the tube. During the ensuing 10 minutes, the mixture is examined under the microscope against a polished black background. The time required for the formation of flocculi gives a rough index of the concentration of protein present. Thus frog plasma, diluted 1:100, gives a heavy precipitate within 1 minute; a 1:700 dilution shows a few flocculi within 7 minutes. In dilutions lower than 1:100 the precipitate was very gross; in dilutions greater than 1:700, protein could not be consistently detected.

Trichloroacetic acid is a more convenient reagent than acetic acid and potassium ferrocyanide but no more sensitive. Concentrated nitric acid (used by Ekehorn) in our experience has proved insensitive and unreliable in the detection of small amounts.

The consistent accuracy with which the manipulations above described have been applied to the analysis of volumes of fluid of from 0.03 to 0.5 c.mm., containing dissolved substance in quantity from a millionth to a few hundred-thousandths of a mg., will be apparent in tables of control analyses in the papers which follow this. It is not unduly difficult to acquire by practise the manipulative skill required for a high degree of accuracy. The most important requirements are never ceasing watchfulness for flaws in technique and willingness to retrace and repeat any step of doubtful integrity.

#### SUMMARY

A manipulative technique is described for adapting current methods of quantitative colorimetric determination of certain constituents of blood and urine to volumes of fluid less than 1 cubic millimeter. It has been designed for application to problems of the quantitative composition of fluids obtainable from single structural units of the kidney. It is believed to be applicable to many other biological problems.

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## QUANTITATIVE STUDIES OF THE COMPOSITION OF GLOMERULAR URINE

### VIII. THE CONCENTRATION OF URIC ACID IN GLOMERULAR URINE OF SNAKES AND FROGS, DETERMINED BY AN ULTRAMICROADAPTATION OF FOLIN'S METHOD\*

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This work was undertaken because of a desire to determine the concentration of uric acid in the glomerular urine of snakes. Previous analyses of glomerular urine, collected from amphibian kidneys only, had yielded conflicting evidence concerning the existence of secretory processes within the glomerular membrane. Some of these seemed to show that the chloride concentration and the total molecular concentration exceed those of blood plasma sufficiently to indicate glomerular secretion. These results were difficult to believe because determinations of other constituents did not agree with them and because of the seeming uselessness of a process whereby chloride is concentrated in glomerular urine in a kidney which excretes almost no chloride.

We proposed to look for evidence of glomerular secretion in an animal in which that process could be conceived to be useful. Snakes possess the capacity of restricting water excretion to a marked degree. Concentration of the urine in the cloaca by reabsorption of water constitutes an important item in their water

\* The expenses of this investigation have been defrayed in large part from a grant by the Commonwealth Fund. Preliminary reports of these experiments were made before the National Academy of Sciences, April 25, 1932, and before the American Society of Biological Chemists at Philadelphia, April 29, 1932 (*J. Biol. Chem.*, **97**, lxxii (1932)).

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economy. Uric acid is the chief solid constituent of snake urine. It was thought that glomerular secretion, if it exists in any animal, might be revealed in the snake as one of the means whereby it eliminates uric acid with the least possible attendant loss of water.

The desirability of the study was further increased by the consideration that thus far the microscopic observation of glomerular phenomena during life and microanalytical studies of glomerular urine have been restricted to the amphibian kidney, a mesonephros. Clearly it is of interest and possible importance to extend this type of investigation to land animals in which the kidney has developed to the stage of a metanephros.

In Paper VII of this series (1), an account is given of the manipulative technique which was developed for accurate quantitative determination not only of uric acid but of other constituents of blood and urine by capillary tube colorimetry. Section I of the present paper contains additional technical details which apply only to the determination of uric acid, together with results obtained when the method was applied to the analysis of fractions of a c.mm. of known uric acid solutions.

Section II contains a description of experiments with frogs. They were injected with small amounts of uric acid in order to raise the blood level of this substance to the range required by the analytical method; glomerular urine and blood plasma were collected, analyzed, and their uric acid concentrations compared. These experiments were undertaken not only because of interest in glomerular function of the frog, but primarily because experience in the application of a new method could more easily be obtained by the use of material from a familiar and readily accessible source.

Section III contains a description of six successful experiments with small snakes in which glomerular fluid was collected, analyzed, and its uric acid concentration compared with that of blood plasma.

No evidence has been obtained, either in snakes or frogs, which supports the thought that any process other than filtration is responsible for the glomerular separation of uric acid.

In the past, direct evidence of the passage of uric acid through the glomerular membrane has been sought chiefly in the microscopic study of sections of kidneys of animals which had received intravenous injections of uric acid shortly before they were killed.

Working with rabbits, Ebstein and Nicolaier (2), Sauer (3), Aschoff (4), and Eckert (5) found no uric acid in the capsular spaces; Schultz (6), however, did. Cordier (7) studied sections of reptilian kidneys and found uric acid in the capsular spaces only when amorphous material (protein) was also present. From this she concluded that uric acid is not excreted through the glomerulus in normal kidneys. Her view has been accepted by Holton and Bensley (8). Lueken (9) injected a frog with lithium urate; he found no evidence of its presence in the capsular spaces of the sections which he studied. On this histological evidence one would scarcely be willing to base a firm belief concerning glomerular excretion of uric acid.

Lueken's (9) chief approach to the question was by the Cullis method of double perfusion of the frog kidney as it has been adopted in modified form by Hober and his pupils. When lithium urate was added to the aortic perfusion fluid only, it appeared in the urine in concentration 2 to 2.5 times that of the perfusion fluid. The conclusion was drawn that uric acid passes through the glomerulus.

It seems superfluous to cite here work on the excretion of uric acid in which glomerular filtration of a fluid identical in uric acid concentration with plasma is an accepted premise. The correctness of this premise is the chief concern of this work.

### *I. Analytical Method*

Folin's colorimetric method for the determination of uric acid has been adapted to volumes of fluid ranging from 0.03 to 0.5 c.mm. containing from 3 to 10 millionths of a mg. of uric acid. The reactions and color comparisons are conducted in capillary tubes; the details of apparatus and manipulations required in such refinement of colorimetry have been described in Paper VII of this series (1). Those which particularly concern uric acid are as follows:

*Standard Solutions*—The standard uric acid solution of Folin (1 cc. contains 1 mg.) was made according to his directions (10). From this was prepared, in 25 cc. volumetric flasks, a series of seven dilute standards: 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, and 2.0 mg. per 100 cc. It is within this range of concentrations that the intensities of color produced in small capillaries are most reliably comparable.



*Reagents*—The cyanide-urea solution and the uric acid reagent were prepared precisely according to Folin's most recent directions (Folin and Marenzi (11)).

*Procedure*—Into a capillary tube of uniform caliber (0.35 mm.) were successively introduced three columns of fluid of measured length, separated by columns of air, as follows: uric acid solution, 5 mm.; cyanide-urea solution, 5 mm.; uric acid reagent, 1 mm. The ends of the portion of the tube containing them were sealed. After all the tubes of a series (unknowns and seven standards) had been prepared and sealed, mixture of the fluids in each tube was effected simultaneously in all by centrifugation. The exact time of mixing was noted; 4 minutes later the tubes were immersed for 1 minute in boiling water. After identification and convenient arrangement of the tubes, their contents were transferred to other capillary tubes, the inner diameters of which were known by measurement to be the same. Comparisons of the colors of the unknown solutions with those of the standards were then made, usually by two observers independently, each ignorant of the other's readings. The average of the two readings was the result accepted.

In only two respects, aside from apparatus and quantities employed, does this procedure differ from that of Folin. The time of heating was 1 minute instead of 2 because of the rapidity with which the contents of a capillary tube take the temperature of the liquid in which the tube is immersed. Final dilution of the mixture after the color had developed in it was omitted.

The blue color which develops when a solution of uric acid is mixed with reagents and heated in a capillary tube is more intense than that produced when the conventional amounts of the same solutions and reagents are mixed in exactly the same proportions and heated for 2 minutes in a test-tube. It is for this reason that the standard colors were developed in capillary tubes.

The color comparisons were made over the unglazed surface of a milk glass plate illuminated by two 100 watt bulbs. The lengths of columns of fluids compared were equalized by the use of a paper shield in which a rectangular window was cut. Agreement between the color comparisons made independently by the two observers was usually very good: of 185 pairs of consecutive readings 81 were alike, 37 differed by 0.05, 55 by 0.1, 4 by 0.15, 7 by 0.2, and 1 by 0.25 mg. per 100 cc.

The standard solutions differ from each other by 0.2 mg. per 100 cc. At the low end of the range this difference amounts to 33 per cent; at the high end, 14 per cent. When the color developed in a tube of unknown failed to match any standard exactly, the difference was estimated to the nearest 0.05 mg. If it is assumed that this was done correctly, the possible reading error at the low end of the range was about 4 per cent. It is surprising, perhaps, that the average error of the method as a whole is less than 5 per cent. This can be explained by the facts that the practised eye becomes extraordinarily skilful in estimating degrees of color difference and that we usually succeeded in so diluting the unknown that its concentration fell in the middle of the range of standards, *i.e.* 1.0 to 1.4 mg. per 100 cc., where the difference between two standards is 20 per cent or less.

*Protein-Free Plasma Filtrates*—In all of the experiments described here, protein-free plasma filtrates were obtained by precipitation with tungstic acid in capillary tubes and centrifugation, in the manner described in Paper VII of this series (1). Analysis of these filtrates for uric acid was conducted by the capillary tube method.

### *Tests of Method*

50 lithium urate solutions have been analyzed, forty-seven in duplicate, one in triplicate. In twelve the concentration of the solution to be determined was known to the analyst. The results of these are not represented in the tabulation because the criticism might be made that a psychological factor influenced the result. Eight of the remaining analyses have been discarded because they were made in the early stages of the work and contained sources of error which were subsequently eliminated (color comparison in tubes of unequal bore, omission of the use of oil to prevent evaporation, faulty preparation of the standard tubes). This leaves thirty control analyses of known watery solutions the results of which are summarized in Table I. The concentration of these solutions was unknown to the analyst while he was making the determination.

In every case the volume of uric acid solution which was mixed with the reagents was about 0.5 c.mm. (*i.e.*, a 5 mm. column in a tube of 0.35 mm. bore). In the majority of analyses, the original

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fluid to be analyzed required dilution to bring its concentration into the concentration range of the standards. The highest dilution required in our series of controls was 20-fold. Hence, it is correct to say that we have determined the uric acid in as little as 0.025 c.mm. of solution. The absolute amounts of uric acid determined varied from 3 to 10 millionths of a mg. The approximate amount in any one analysis can be calculated from the orig-

TABLE I  
*Representative Determinations of Uric Acid Concentration of Known Solutions of Lithium Urate*

Volume of solution analyzed, approximately 0.5 c.mm.; absolute amounts of uric acid, 3 to  $10 \times 10^{-6}$  mg.

Experiment No.	Dilution of solutions analyzed	Concentration of solution		Difference per cent	Typical of
		Found*	Known		
		mg per 100 cc.	mg per 100 cc		
10	1.5, 2.5	3.2	3.2	0	3 experiments
14	7 1, 4 8	5.05	5.0	+1	17 experiments; errors 1 to 5 per cent. 9 plus; 8 minus
28	19 4, 20 0	26.55	25.8	+3	
2	0, 0	1.05	1.0	+5	
12	3 2, 4 4	3.9	4.0	-3	
29	20, 19 3	26.5	27.4	-3	7 experiments; errors 6 to 8 per cent. 4 plus; 3 minus
19	15, 15	11.65	11.0	+6	
15	6 9, 5 5	6.0	6.4	-6	
3	0, 1 9	1.35	1.2	+12	3 experiments; errors 11 to 14 per cent. 2 plus; 1 minus

\* Average of duplicates.

inal concentration of the solution, its dilution, and the dimensions of the column taken for analysis given above.<sup>1</sup>

Table I is representative of 59 separate estimations. Of these ten

<sup>1</sup> Following are the details of a typical analysis. A uric acid solution containing 10.0 mg. per 100 cc. was made by A. N. R. About 0.5 c.mm. was taken into the tip of a quartz collecting pipette. Into capillary Tube A was discharged under oil a column of this solution 2.41 mm. long. A water column 6.25 mm. long was then introduced and the two columns thoroughly

were correct; in thirty-five (59 per cent) the error was 5 per cent or less; in 52 (88 per cent) it was not greater than 10 per cent; in only seven was the error between 10 and 20 per cent. The mean of these errors, the sign being taken into account, was  $+0.13$ ; the average deviation from the mean, 5.1 per cent. When the averages of duplicates are considered, in twenty (67 per cent) the error is 5 per cent or less; in twenty-seven (90 per cent) it is 8 per cent or less; in three it is from 11 to 14 per cent. The mean of these averages is  $+0.5$  per cent; the average deviation from the mean, 4.5 per cent. From these results we conclude that the method applied to these minute quantities is amazingly reliable.

*Tungstic Acid Filtrates*—These were prepared from frog plasma

mixed by centrifugation (Solution A) Into capillary Tube B were similarly introduced and mixed 1 72 mm of the uric acid solution and 5 80 mm. of water (Solution B).

*Duplicate Analyses of Solutions A and B*

Solution	Lengths of columns in capillary tube, 0.35 mm bore			
	Uric acid solution	Water	Cyanide-urea	Uric acid reagent
	mm	mm	mm	mm
A <sub>1</sub>	1 5	3 5	5 0	1 0
A <sub>2</sub>	1 8	3 2	5 0	1 0
B <sub>1</sub>	2 55	2 50	5 05	1 0
B <sub>2</sub>	2 0	3 0	5 0	1 0

*Color Comparisons with Standard Solution, Mixed and Heated Simultaneously with Four Unknowns*

Solution	Readings (mg per 100 cc of final dilution)			Calculations (mg per 100 cc of original solution)	
	A N R	J B	Average		
A <sub>1</sub>	0 8	0.8	0 8	$0.8 \times 866/241 \times 50/15 = 9.6$	9.8 Average 10.65 "
A <sub>2</sub>	1 0	1 0	1 0	$1.0 \times 866/241 \times 50/18 = 10.0$	
B <sub>1</sub>	1 2	1 3	1 25	$1.25 \times 752/172 \times 505/255 = 10.9$	
B <sub>2</sub>	1 0	0 9	0 95	$0.95 \times 752/172 \times 50/20 = 10.4$	

J. B. was ignorant of the strength of the original solution and of the first dilution; he made a preliminary estimation to decide on the appropriate second dilution. A. N. R. was ignorant of the second dilution.

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which had been dialyzed for 48 hours against a sodium chloride-sodium oxalate solution. After dialysis known amounts of uric acid were added (unknown to analyst). Table II contains the results of analyses of nine samples of plasma, each made in duplicate. In three of these (Experiments 6, 8, and 9) 0.5 cc. of plasma was precipitated according to Folin and Wu; in the others the precipitation was made in large capillary tubes according to the capil-

TABLE II  
*Determinations of Uric Acid in Tungstic Acid Filtrates from Dialyzed Frog Plasma to Which Uric Acid Was Added in Known Concentration (Unknown to Analyst)*

Experiment No	Date	Dilutions of plasma	Concentration of solution			Difference
			Found		Known	
			Duplicates	Average		
	1931		mg per 100 cc	mg per 100 cc	mg per 100 cc	per cent
1	Oct. 10	2 5, 2 5	1 6, 1 8	1.7	1.7	0
2	" 10	2 5, 2 5	2 3, 2 3	2.3	2.1	+10
3	" 10	2 5, 2 5	2 9, 3 0	2.95	2.8	+5
4	" 10	2 5, 2 5	3 1, 3 1	3.1	3.2	-3
5	" 12	5 0, 5 5	4 1, 4 4	4.25	4.6	-8
6	July 2	5 6, 5 6	5 3, 5 1	5.2	5.3	-2
7	Oct. 12	5 0, 5 0	6 0, 6 0	6.0	5.8	+3
8	July 1	10 0, 10 0	8 0, 8 5	8.25	8.3	-1
9	June 30	17 0, 17 0	8 9, 9 8	9.35	9.1	+2
Mean						+0 7
Average deviation from mean						±3 8

lary tube method described in Paper VII of this series (1). In these the volume of plasma taken was 1.5 to 2.0 c.mm.

The greatest individual errors among these eighteen separate estimations are two of +10 per cent; one of -11 per cent. In eleven the error was less than 5 per cent; in four, 6 to 8 per cent. If the average of duplicates is taken as the accepted result, in six the concentrations found were within 4 per cent of the true value; only one was as far wrong as 10 per cent, and in this instance the difference amounted to only 0.2 mg. per 100 cc.

The results show that the manipulations incident to dilution of the plasma and precipitation of the proteins do not introduce appreciable added error.

## *II. Experiments with Frogs*

*Kidney Perfusions*—In five experiments, frog kidneys were perfused via the aorta with artificial solutions to which uric acid in known amount had been added. Perfusion was begun before the natural circulation of blood through the kidney was interrupted. Perfusion pressure was 25 to 35 cm. of water. In Experiments 1 and 2 the perfusion fluid was unoxygenated Clark-Ringer solution; in Experiments 3 to 5, it consisted of sterile normal horse serum, 1 part, and Clark's solution, 2 parts, with glucose added to make 0.06 per cent, urea to make 0.02 per cent, and sodium bicarbonate to make 0.05 per cent, the final fluid being thoroughly oxygenated. In each experiment glomerular fluid was collected from a single renal corpuscle. In Experiments 1 and 2 no effort was made to obstruct the tubule; in Experiments 3 to 5 the tubule was identified by injection of phenol red and effectively blocked close to the glomerulus by compression with a glass rod. The results are shown in Table III.

These results show clearly that the uric acid concentration of glomerular urine taken from a perfused kidney is the same as that of the perfusion fluid. The result is the same whether the kidney is perfused with unoxygenated protein-free Clark's solution or with oxygenated protein-containing fluid. With respect to uric acid the glomerular fluid in these experiments is a filtrate. The concentrations of uric acid in the bladder urine which accumulated during the perfusion of Experiments 1 and 2 were 2 and 3 times those of the glomerular fluids. This shows that the vitality of the kidney had not been abolished.

*Uric Acid in Glomerular Urine and Plasma from Living Frogs*—The technique employed in preparing the animal and collecting glomerular urine has been described in previous publications from this laboratory. A brief résumé is as follows: The frogs used were *Rana pipiens*, ♀, weights 50 to 70 gm. Half an hour before the beginning of the operation, 1 to 4 mg. of uric acid (as lithium urate) were injected into the anterior lymph sac. The brain was crushed with a hemostat. An electric cautery was used for dissection;

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usually there was no hemorrhage. The right kidney was exposed and transilluminated. Bowman's capsule was punctured with a quartz pipette, the tip of which was filled with saline containing 0.15 per cent phenol red. The tubule was identified by injection of the dye and blocked with a glass rod after all the dye solution

TABLE III  
*Uric Acid in Glomerular Fluid from Perfused Frog Kidneys*

Experiment No	Date	Concentration of uric acid in				Difference	
		Perfusion fluid		Glomerular urine			
		Duplicates	Average	Duplicates	Average		
	1931	mg per 100 cc	mg per 100 cc	mg per 100 cc.	mg per 100 cc	per cent	
1	June 2	4 5, 4 1	4.3	4 3, 4 1	4.2	-2	Bladder urine, 8.7 mg per cent
2	" 7	4 6, 5 3	4.95	5 0	5.0	+1	Bladder urine, 15.9 mg per cent
3*	Sept. 17	6 2, 6 0	6.1	6 3, 6 5	6.4	+5	Protein present in glomerular urine
4*	" 19	5 9, 6 6	6.25	5 9, 6 3	6.1	-2	No protein in glomerular urine
5*	" 19	5 9, 6 6	6.25	6 7	6.7	+7	Same preparation as in Experiment 4. No protein in glomerular urine

\* These collections were made by Dr. Walker primarily for the study of the passage of reducing substances through the glomerulus (see Paper IX of this series (12)).

had been injected and the tip of the pipette had been washed four to six times with freshly formed glomerular urine.<sup>2</sup> Mercury pres-

<sup>2</sup> We long ago abandoned the device, originally used by Wearn and Richards and retained in the recent work of Ekehorn (13), of drying the surface of the kidney before puncturing Bowman's capsule. Its purpose was to prevent contamination of the tip of the pipette by fluid on the surface of the kidney. The purpose is far more conveniently and effectively accomplished by repeated washing of the tip of the pipette with glomerular fluid immediately after puncture, before beginning collection of glomerular fluid. This is done by alternately lowering and raising the mercury leveling bulb.

sure in the collecting pipette system at the beginning of collection was over 200 mm. The mercury bulb was lowered gradually to about 30 mm. above the level of the kidney, then still more gradually to +10 to +5 (this to avoid suction due to capillarity of the pipette).

Blood samples (each about 0.02 cc.) were taken from the ventricle of the heart into capillary pipettes containing a few grains of dry sodium oxalate.

Glomerular urine immediately after collection was transferred under the microscope into a capillary tube of uniform bore, the narrowed<sup>3</sup> end of which contained a short column of oil. The tip of the pipette projected into the oil so that the column of glomerular urine was never exposed to air. A column of distilled water of suitable length was then introduced, separated from the glomerular urine by a column of oil. The fluids were drawn further into the tube, the distal part of the tube containing them broken off, its ends sealed, and the lengths of the columns of glomerular urine and water accurately measured. Mixture of glomerular urine and water was effected by repeated centrifugations. When a second dilution was necessary it was made in the mixing capillary.

The preliminary determinations required to indicate the degree of dilution necessary to bring the uric acid concentration of plasma and glomerular urine into the range of the standard solutions were made on the protein-free tungstic acid filtrates of plasma, the supply of which was always abundant. The close agreement between the uric acid values of plasma and glomerular urine made separate preliminary estimations on the latter unnecessary.

Between April 1 and December 1, 1931, forty-four experiments were made. We shall publish the results of only thirteen. The reason for excluding such a large number is the fact that they were made before we had mastered all of the details of analytical technique essential for reliable results. The chief difficulties were encountered in analysis of plasma. A few experiments were ruined by partial hemolysis of blood samples from careless use of too much oxalate. In eleven of the discarded experiments, uric acid in plasma

<sup>3</sup> The receiving capillary is mounted on the glass stage of the microscope at an angle with horizontal. Unless the extreme end of the tube is constricted either by drawing it out or by holding it in the flame for an instant, the column of oil will flow back from the tip.



was determined in an ultrafiltrate made by filtration under oil through cellophane. Discrepancies in results occurred which led to the discovery that cellophane holds back varying fractions of the urates in plasma. This does not, however, show that part of the plasma urate is "bound;" membranes made from parlodion give protein-free ultrafiltrates having the same concentration of uric acid as the plasma. For a time it seemed that the presence of a small amount of protein did not influence the uric acid estimation; that if the uric acid concentration of the plasma were such as to require more than 4-fold dilution to bring it within the standard range, removal of protein might be omitted. Several experiments were made on the basis of this belief. More scrupulous tests showed that we were wrong; that the blue color from reduction of the phosphotungstic acid is intensified by the presence of protein. It was then that we developed the method of obtaining protein-free plasma filtrates by precipitation with tungstic acid in capillary tubes. Ample evidence has been obtained by others to show that this macromethod applied to *plasma* gives correct results. Our experience has convinced us that equally satisfactory results are consistently obtained with it by the micromethod. This adaptation of technique was accomplished between October 1 and October 10, and we have decided to discard all of our experiments on living frogs made before the latter date. Since then, sixteen experiments have been made. Three of these have been discarded: one because it was practically certain that the glomerular urine became admixed during collection with fluid from the surface of the kidney; another because the water used in diluting the glomerular urine was found to contain traces of chlorine (it was improperly distilled from the city water supply which is chlorinated); and the third because 2 days elapsed between the collection and the analyses of glomerular urine and plasma.

The details of the experiments and the results are brought together in Table IV. Descriptive comment on the conduct of individual experiments is unnecessary. In all, the circulation was vigorous and the details of technique satisfactory. For convenience, the following summary of percentage differences between the uric acid of plasma and that of glomerular urine is given.

Experiment No...	29	32	42	39	41	44	37	40	43	31	36	33	38
Difference, per cent	0	0	0	0	+4	+4	-5	-6	+6	+13	-15	-17	+22

TABLE IV

*Uric Acid in Glomerular Urine and Blood Plasma of Living Frogs*

Experiment No and date (1931)	Glomerular urine			Time of collection		Concentration of uric acid in					Difference	Protein test†
	Time of collec- tion	Volume	Collection pressure	Blood 1	Blood 2	Plasma 1	Plasma 2	Average	Glomerular urine			
	min	cc mm	mm Hg	min *	min *	mg per 100 cc	mg per 100 cc	mg per 100 cc	mg per 100 cc	per cent		
29 Oct. 12	50		+6	12	39	1 5 1 5	1 6 1 6	1 6	1 6 1 5	1.6	0	+
33 Oct. 16	52	0 5	+9	17	35	1 75 1 75	1 9	1.8	1.5		-17	0
37 Nov 4	62	0 3	+8	25	62	2 1 2 1	1 8 1 8	2.0	1.9		-5	
31† Oct 14	58	0 9	+10	5	53	3 0 2 9	3 4 3 5	3.2	3.6		+13	?
32† Oct 14	71	0 8	+6	5	65	3 3 3 1	3 2 3 2	3.2	3 2 3 1	3.2	0	?
40 Nov 6	60	0 4	+11	12	67	3 75 3 75	3 3 3 4	3.6	3 6 3 2	3.4	-6	+
36 Nov 2	47	1 3	+11	13	47	4 0 3 8	4 0 3 6	3.9	3 3 3 1 3 4	3.3	-15	0
42† Nov 10	84	0 7	-2	10	75	4 7 4 4	4 2 3 95	4 1	4 2 4 3	4.3	0	?
41† Nov. 9	85	0 8	+2	10	75	5 0 5 0	4 4 4 4	4.7	5 0 4 75	4.9	+4	+
38 Nov 4	90	0 07	+10	20	91	4 95 5 1	4 75 4 75	4.9	6 0 6 0	6.0	+22	0
39 Nov 5	80	0 5	+10	19	81	5 8 5 6	4 4 4 8	4 6	5 1 5 3	5.2	0	+
44† Dec 1	36	0 9	+7	9	30	5 6 6 0	5 0 5 0	5.4	5 6 5 6	5.6	+4	+
43† Nov 30	39	0 8	+5	5	35	10 2 9 4	8 1 7 5	7 8	9 0 9 7	9.4	+7	0

\* After the beginning of collection of glomerular urine.

† See p. 207.

‡ These collections were made by Dr. Walker primarily for determination of reducing power (see Paper IX). The animals received preliminary injections of uric acid and portions of the fluids collected were given to us for uric acid analysis

When it is considered that each of these differences is calculated from analyses of two samples of plasma and one of glomerular urine, it is obvious that in nine experiments the results indicate identity of uric acid concentration of plasma and glomerular urine; in the other four, the differences, two plus and two minus, are not too great to be ascribed to the sum of experimental and analytical errors. From this evidence we conclude that uric acid passes through the glomerular membrane of the frog in the same concentration as that in which it exists in the water of the blood plasma.

*Concentration Ratios and Plasma Uric Acid Clearance Values—*  
In only three of the experiments cited above has urine from ureter or bladder been collected and analyzed. Two of these were Experiments 1 and 2 of the perfusion series; the ratios of urinary to plasma uric acid in these were 2.0 and 3.2. The third was Experiment 32 of the series on living frogs, in which bladder urine was taken at the end of the experiment; in this the ratio was 3.75. Seven other experiments have been made for the especial purposes of determining the concentration ratio and of calculating the plasma uric acid clearance. In these, the frogs were injected with 2 to 9 mg. of uric acid as lithium urate; after half an hour the brain was crushed and the bladder emptied by catheter; from 30 to 80 minutes later the urine was collected by catheter and blood drawn as quickly as possible from the aorta. From volume of urine and concentrations of uric acid in urine and plasma are calculated not only the concentration ratios and plasma clearances but also the rate of plasma uric acid clearance per glomerulus per hour, the assumptions being made that all of the uric acid excreted was filtered and that 2000 glomeruli in each kidney were active (14). The results are given in Table V. The figures show (a) that in the perfusion experiments the concentration ratios are like those recorded by Lueken in which uric acid was supplied to the kidney only by way of the arterial circulation (2.0 and 3.2 *versus* 2.0 and 2.5); (b) in no case did the concentration ratio reach the average of 10 observed by Lueken when uric acid was supplied to the kidney by the renal portal vein only; nor did it approach his high figures (17.5 and 22). (c) If it is assumed that each kidney contained 2000 glomeruli and that all were active, the highest rate of glomerular filtration necessary to separate all of the excreted uric acid from the blood was 0.45 c.mm. per glomerulus per hour, a figure well within

the range of rates of glomerular urine collection which were observed in the experiments of Table IV and much less than rates of collection which have occasionally been made. While there is no evidence in these figures to show that some portion of the uric acid excreted was not secreted by the tubules, there is obviously no necessity for assuming that this was the case.

*Protein in Glomerular Urine*—In these experiments glomerular urines have been tested for the presence of protein in more systematic fashion than in earlier experiments from this laboratory. The procedure outlined in Paper VII of this series (1) was used. In most of the experiments the glomerular urine was diluted before

TABLE V

*Uric Acid in Plasma and Bladder Urine of Pithed Frogs. Concentration Ratios and Plasma Uric Acid Clearance*

Experiment No	1	2	3	4	5	6	7
Uric acid injected, <i>mg</i>	2	2	2	4	4	8	9
Time of urine collection, <i>min</i>	70	32	35	75	34	81	35
Volume of urine, <i>cc</i>	0 74	0 13	0 05	0 07	0 6	0 01	0 04
Uric acid in urine, <i>mg per cent</i>	28 9	20 9	41 2	46 5	25 7	185	127
“ “ “ plasma, “ “ “	15 0	4 96	5 05	14 7	15 2	27 1	23 2
Ratio, <i>U P</i>	1 9	4 2	8 2	3 2	1 7	6 8	5 5
Plasma uric acid clearance per hr, <i>cc</i>	1 22	1 02	0 70	0 18	1 8	0 05	0 38
Filtrate per glomerulus per hr, assuming 4000 active, <i>c mm</i>	0 3	0 25	0 17	0 04	0 45	0 01	0 1

being tested. In three experiments (Experiments 33, 36, 43) the urines, diluted from 1.8 to 2.9 times gave no detectable reaction. In three others (Experiments 29, 42, 44) the protein content, calculated for undiluted glomerular urine, was less than that in frog plasma diluted 200 times. In the others it was about equivalent to that in plasma diluted from 150 to 200 times.

### *III. Experiments with Snakes*

Preliminary experiments made some years ago by one of us showed that glomeruli in the kidney of the living snake are visible under the microscope, and that the preparation of the kidney for microscopic study and manipulation is scarcely more difficult in

the snake than in the frog. The circulation in a snake kidney as a rule is maintained during a long experiment with greater vigor than in the frog.<sup>4</sup> The length of the renal arteries is such that the kidney can be drawn out of the body cavity and placed on the window of the animal board without recognizable disturbance to the renal blood supply. When the ventral surface of the kidney is examined, glomeruli are seen arranged in irregular rows, parallel with and lateral to the conspicuous renal vein. The renal corpuscles, elliptical in shape, and the glomeruli in them are smaller than in the frog; the free capsular space is considerably smaller both absolutely and relatively—a fact which makes puncture of the capsule without damage to the glomerulus difficult. Measurements of a number of glomeruli in brown snakes gave figures for the lesser diameter ranging from 120 to 159 $\mu$ , for the greater diameter 140 to 210 $\mu$ .<sup>5</sup> Sections made for us by Dr. Balduin Lucké showed that the glomerular epithelium is more conspicuous than in frogs or mammals.

In a number of instances we have seen that the afferent arteriole divided into two branches immediately after its entrance into the capsule; in others, the circulation of blood ceased in one part of the glomerular capillaries, corpuscles remaining stagnant, while it continued in the rest. From this we conclude that in these instances at least the glomerulus did not consist of a single coiled capillary as Bowman (16), Heidenhain (17), and more recently Regaud and Policard (15) believed. The capillaries seem to be coiled about the surface of a transparent core, as described by Regaud and Policard.

The neck of the tubule proceeds laterally from the pole of the renal corpuscle opposite the entrance of the afferent vessel. The efferent vessel often runs parallel with and close to this so that ob-

<sup>4</sup> In one experiment a snake weighing 12.5 gm. was anesthetized with urethane at 12 noon, the kidney exposed, and the total number of visible glomeruli in the left kidney counted. The number was 140; circulation was active in all. The snake was left on the board all night in the warm, dry air of the laboratory, no precautions being taken to prevent drying. The next morning, 22 hours after the anesthetic was given, circulation was fairly vigorous in 70 visible glomeruli.

<sup>5</sup> These figures are larger than those cited by Regaud and Policard (15) for the glomeruli of *Tropidonotus natrix* and *Tropidonotus viperinus* and *Vipera aspis*. Their measurements were not made on living material.

struction of the neck of the tubule by pressure with a glass rod may result in obstruction of the glomerular circulation.

All of the uric acid in snake blood plasma is filtrable through collodion (parlodion) membranes. In one experiment, 0.4 cc. of protein-free ultrafiltrate was obtained from 1.8 cc. of fresh, oxalated plasma; in another, 0.4 cc. of protein-free fluid was filtered from 1.0 cc. of plasma. Analyses of ultrafiltrates and fluid left in the filter were made by Folin's method.

	Residue mg per cent	Ultrafiltrate mg per cent
Experiment 1	14 2	14 1
" 2	13 6	13 9

*Experimental Technique*—Snakes of three species have been used: the garter snake, *Eutænia sirtalis*, the brown snakes, *Storeria occipitomaculata* and *Storeria dekayi*. Female snakes were preferred because in males the sexual segments of the tubules obscure many of the glomeruli; the greater thickness of the male kidney makes both illumination and puncture more difficult.

As anesthetic urethane (2.5 to 10 per cent solution) was given by stomach tube in dosages of 3 to 5 mg. per gm. In some instances dosage was uncertain because of regurgitation. Artificial respiration, when necessary, was given through a tracheal cannula by a small Palmer oscillating pump.

The body cavity was opened by a mid-line incision beginning a little above the vent and extending to the level of the upper pole of the left kidney. The cut edges of the skin were pinned down, the ribs of the left side were cut away with a cautery, the membranes which bind the kidneys to the intestine were torn apart with fine forceps, and the left kidney lifted out of the body cavity so that the caudal portion could be laid upon the glass window of the operating board without producing tension on the vessels. The kidney was held in position by pins thrust through the oviduct. The operation, properly done, is practically bloodless. During the period of the experiment the caudal portion of the snake was fastened to the board which we have used in glomerular punctures in frogs; the neck and anterior part of the body were fastened by pins to another board supported at the same height as the microscope stage.

The kidneys of the smaller snakes were illuminated by the transmitted light from a Zeiss incandescent microlamp filtered through

copper sulfate-picric acid solution; when larger snakes were used the beam from a carbon arc, similarly filtered, was focused directly on the ventral surface of the kidney.

The method of puncture of Bowman's capsule was essentially the same as that first used by Wearn and Richards (18) in work on the frog. The thickness of the kidney, its yielding character, together with the toughness of the peritoneum and Bowman's capsule and the small dimensions of the capsular space make puncture far more difficult than is the case in frogs. The quartz pipettes with very slender tips previously used routinely in work with frogs are not rigid enough to be used with advantage in piercing the tougher membranes of the snake. For this reason we have resorted to pipettes made from Pyrex glass, the tips of which are drawn at a less acute angle. The average diameter of the extreme tip was approximately  $8\mu$ . Blood taken during the course of a collection of glomerular urine was drawn from a carotid artery, exposed during the preliminary operations. Determinations of uric acid in glomerular fluid and plasma were made by the capillary tube technique described above and in Paper VII of this series.

In four experiments a cannula was inserted into the ureter of the kidney which was being studied. In three of these the rate of urine formation was measured during part of the period of glomerular urine collection. Uric acid in the urine was determined by Folin's method.

In four experiments the total number of glomeruli in one or both kidneys was counted by Nelson's method (19).

*Experimental Details and Results*—In six experiments only have we succeeded in collecting enough glomerular urine for analysis. Many failures have been due to the difficulty of making a perfect puncture without injury to the glomerulus. The results of the analyses and such details of the experiments as are suitable for tabulation are contained in Table VI. Complete analytical data are contained in Table VII. Details unsuitable for tabulation but important in judging the experiments are as follows:

*Experiment 1*—November 24, 1931. Natural respiration throughout experiment. Cannula in ureter. Technique of puncture perfect. Tubule partially blocked. Mercury in leveling bulb never lower than +4 mm. Glomerular urine collection begun  $4\frac{1}{2}$  hours after injection of urethane.

Blood flow ceased in part of tuft during latter part of glomerular urine collection.

*Experiment 2*—November 30, 1931. Artificial respiration during the period of glomerular urine collection. Pipette in excellent position in capsular space throughout collection. Tubule not blocked Glomerular

TABLE VI

*Uric Acid in Blood Plasma, Glomerular Urine, and Ureteral Urine of Snakes*

Experiment No and date (1931-32)	Species* and weight of snake	Glomerular urine		Time of blood samples	Concentration of uric acid in			No of glomeruli in kidney	Rate of urine flow
		Time of collection	Approximate volume		Plasma	Glomerular urine	Ureteral urine (concentration ratio)		
	gm	min	c mm	min †	mg per 100 cc	mg per 100 cc	mg per 100 cc		cc per hr
1 Nov. 24	<i>S. o.</i> ♀ 11	95	0 23	97	12.2	11.9	279 (23)	R. 1,413 L. 1,471	
2 Nov 30	<i>E. s.</i> ♂ 66	50	0 25	60	10.6	9.9	111 (10 5)	R. 8,356 L. 7,743	0 24
3 Dec 14	<i>E. s.</i> ♀ 68	170†	0 05	20 180	19.7 20.3	21.4	179 (9)	R. 9,465 L. 9,253	0 29
4 Dec. 21	<i>E. s.</i> ♀ 82	111	0 07	101	9.6	11.4	215 (22)	L. 17,001	0 15
5 June 8	<i>S. d.</i> ♀ 12 5	89	0 1	95	16.9	14.4			
6 June 20	<i>S. d.</i> ♀ 16 7	92	0 1	29 99	27.7 30.0	28.3			

\* *S. o.* = *Storeria occipitomaculata*; *E. s.* = *Eutania sirtalis*; *S. d.* = *Storeria dekayi*.

† After the beginning of collection of glomerular urine.

‡ During 45 minutes of this period no fluid was collected because of obstruction to tip of pipette.

collection begun 4½ hours after first injection of urethane. Pressure in collecting system never lower than +8 mm. Stagnation occurred in part of glomerular tuft late in the experiment; circulation in the rest of the tuft was very vigorous. Cannula in ureter; measured collections of urine made during glomerular urine collection.



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*Experiment 3*—December 14, 1931. Artificial respiration throughout. Carotid artery exposed. Cannula in jugular vein. Cannula in ureter. Renal circulation was poor soon after operation was finished. 2 cc. of

TABLE VII  
*Detailed Data of Uric Acid Analyses of Snake Glomerular Urine and Blood Plasma*

Experiment No	Plasma			Glomerular urine		
	Dilutions	Reading	Concentration*	Dilutions	Reading	Concentration*
		mg per cent	mg per cent		mg per cent	mg per cent
1	(a) 2 5 × 50/10	0 95	11 9	(a) 81/21 × 50 5/15 5	0 9	11 3
	(b) 2 5 × 50/15	1 5	12 5	(b) 81/21 × 50/20	1 3	12 5
			<b>12.2</b>			<b>11.9</b>
2	(a) 2 5 × 50/15	1 2	10 0	(a) 73/21 × 50/24	1 4	10 1
	(b) 2 5 × 50/20	1 8	11 25	(b) 73/21 × 50/20	1 1	9 6
			<b>10.6</b>			<b>9.9</b>
3	I (a) 2 5 × 50/10	1 6	20	45 5/4 8 × 40/30	1 7	21 4
	(b) 2 5 × 50/10	1 55	19 4			
			<b>19.7</b>			
	II (a) 2 5 × 50/10	1 6	20			
	(b) 2 5 × 50/10	1 65	20 6			
			<b>20.3</b>			
4	I (a) 2 5 × 50/10	0 8	10 0	(a) 65 5/8 5 × 50/37	1 1	11 5
	(b) 2 5 × 50/15	1 1	9 2	(b) 65 5/8 5 × 50/29	0 85	11 3
			<b>9.6</b>			<b>11.4</b>
5	I (a) 2 5 × 50/10	1 3	16 25	(a) 55/10 8 × 50 5/20	1 2	15 4
	(b) 2 5 × 50/10	1 4	17 5	(b) 55/10 8 × 50/20	1 05	13 4
			<b>16.9</b>			<b>14.4</b>
6	I (a) 2 5 × 50/7	1 5	26 8	(a) 50/9 4 × 50/12	1 25	27 7
	(b) 2 5 × 50/7	1 6	28 6	(b) 50/9 4 × 50/12	1 3	28 8
			<b>27.7</b>			<b>28.3</b>
	II (a) 4 0 × 50/10	1 55	31 0			
	(b) 4 0 × 50/10	1 45	29 0			
			<b>30.0</b>			

\* Bold-faced figures denote averages.

0.7 per cent NaCl, 2 cc. of 0.7 per cent NaCl containing 1:500,000 adrenalin, and 2 cc. of 0.7 per cent NaCl containing 4 mg. of lithium urate were successively injected intravenously in a space of 2 hours, the last being given

nearly 2 hours before the beginning of glomerular urine collection. Thereafter the circulation was excellent. Measured collections of urine were made during the period of glomerular urine collection. Puncture of Bowman's capsule perfect. Tubule not blocked. Pressure in collecting system slowly lowered to +1 mm. of mercury.

*Experiment 4*—December 21, 1931. Artificial respiration throughout. Cannula in ureter. First collection of glomerular urine, finished 4 hours after first injection of urethane, was discarded because of known accidental contamination with fluid from surface of kidney. Second injection of urethane given 4½ hours after the first. Glomerular urine collection from another capsule begun 34 minutes later. Puncture and insertion of pipette perfect. Tubule not blocked. Pressure in collecting system slowly lowered to +1 mm. of mercury.

*Experiment 5*—June 8, 1932. Artificial respiration. Puncture of capsule and identification of tubule perfect. Block of tubule was complete. Glomerular urine collection began 2 hours 13 minutes after injection of urethane.

*Experiment 6*—June 20, 1932. Natural respiration throughout the experiment. Carotid artery exposed. Puncture of capsule, position of pipette in capsular space, identification and blockage of tubule were all perfect. Glomerular urine collection begun 1 hour 28 minutes after injection of urethane. Mercury leveling bulb was never lower than +8 mm.

In every experiment the circulation in the kidney was excellent during the period of glomerular urine collection. In every experiment also transfer under oil of the glomerular urine from the collecting pipette to the capillary in which the urine was first diluted with water was perfectly made.

Tables VI and VII contain the detailed data and results of the six experiments in which enough glomerular urine was collected to permit analysis. No experiments have been omitted. For convenience, the percentage differences between the uric acid concentrations of plasma and glomerular urine are given.

Experiment No.	1	2	3	4	5	6
Difference, <i>per cent</i>	-2.5	-6.6	+7.0	+18.8	-14.8	-2.0

In four experiments (Experiments 1, 2, 3, and 6) identity of uric acid contents of plasma and glomerular urine within the limits of analytical error is clearly shown. In the other two (Experiments 4 and 5) the divergence was greater; in one of these the glomerular urine appeared to contain more uric acid than did the plasma, in the other less. Because these differences were not in the same direction and because of the length of time required for the col-

lection of the small volumes of glomerular urine, we do not believe that these two results conflict with the conclusion which so clearly issues from the other four; *viz.*, that uric acid concentration of glomerular urine of the snake is the same as that of the blood plasma. It may be objected that in Experiments 1 to 4 the preliminaries to the beginning of glomerular urine collection were unduly prolonged ( $4\frac{1}{2}$  to 5 hours); that progressive deterioration of renal function was occurring during that time. To this it may be answered that no evidence of protein in the glomerular urine was found in any experiment; that the kidney retained its capacity to eliminate concentrated urine during the whole period of the experiments; and that the result of Experiment 6, in which all the preliminaries to glomerular urine collection were promptly finished (1 hour 28 minutes) and in which every detail of the experiment and analyses was as perfect as we can hope to make it, was not only not an exception to the others but exhibited the most perfect agreement between uric acid values of glomerular urine and plasma encountered in the whole series.

*Concentration Ratios and Plasma Clearances of Uric Acid*—Comparison of the uric acid concentration of urine from the ureter with that of plasma in Experiments 1 to 4 gave concentration ratios of 23, 10.5, 9, and 22. They prove that the power of the kidney to concentrate was well maintained under the conditions of the experiments.

In three of these experiments (Experiments 2 to 4) the rate of elimination of urine from the kidney was also measured and the total number of glomeruli in the kidney from which it came was counted. From these figures the average rate of filtration per glomerulus per hour required to account for the uric acid which the kidney actually excreted has been calculated; it was assumed that all of the glomeruli were functioning. In Experiment 2, this calculation gave 0.32 c.mm. per glomerulus per hour; in Experiment 3, 0.28; in Experiment 4, 0.20. Since in Experiment 2 the volume of glomerular urine actually collected from one glomerulus in 50 minutes was 0.25 c.mm., it is clear that these calculations do not yield incredible values. It would be unwise to attempt to draw general conclusions from them; they show only that in these particular experiments it is not necessary to assume that any process other than that of glomerular filtration was responsible for the separation of uric acid from the blood.

We have made attempts to get other evidence on this question. Operative procedures were developed for measuring rate of blood flow through the left kidney of the snake by a method similar to that used by Barcroft and Brodie (20) in rabbits. All of the branches of the aorta posterior to the upper pole of the right kidney were ligated except those which supplied the left kidney. The right renal vein was ligated at the upper pole of the right kidney and clamped just posterior to its junction with the left renal vein; a cannula was inserted into it pointing toward the heart. The vena cava, anterior to the junction, was cleared. At frequent intervals the clamp on the right renal vein was removed, the vena cava clamped, and the time required for 0.1 cc. of blood to flow into the cannula measured with a stop-watch. A minute amount of heparin solution in the cannula prevented clotting. After each measurement, the blood was returned into the cava.<sup>6</sup> Urine from the left kidney was collected by a cannula in the ureter. After adequate experience had been gained with the method, an experiment was made which seemed sufficiently perfect technically to be worth recording.

A female *Eutænia sirtalis*, weight 66 gm., was anesthetized with urethane injected into the stomach. Artificial respiration by negative pressure was maintained by connecting one cylinder of a Meyer respiration pump with the side tube of a body plethysmograph in which the upper half of the snake's body was enclosed. The plethysmograph consisted of a glass tube, somewhat larger in diameter than the snake, over the ends of which rubber dam was fastened. The snake was inserted through holes burned in the rubber dam, small enough to insure air-tightness without interfering with the circulation.

Urine was collected in five periods: Period 1 included the time necessary for the operations; Periods 2, 3, and 5 were 30 minutes each; Period 4 lasted 20 minutes. In the middle of Period 4 the left renal portal vein was ligated. Hence in Period 5 the blood flow measurements represented renal arterial blood flow only.

Blood was taken for analysis at the conclusion of the experiment for determination of cell volume and uric acid in plasma. The

<sup>6</sup>The figure for rate of blood flow in each period is the average of several closely agreeing measurements the number of which is shown in the parentheses of Table VIII.

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total number of glomeruli in the kidney was counted. From these figures and the uric acid values of the urine specimens, the plasma uric acid clearance per hour, the plasma flow per hour, the fraction of plasma filtered, and the average volume of filtrate per glomerulus per hour were calculated. (The last item was calculated for Period 5 only, because only in that period was the renal portal vein ligated.)

The results are given in Table VIII.

TABLE VIII

### *Concentration Ratios and Plasma Clearances of Uric Acid*

January 28, 1932    *Eutamias sirtalis*, ♀, 66 gm    Blood at end of experiment: plasma, 56 per cent; cells 44 per cent. Uric acid in plasma, 14.2 mg. per cent    Weight of kidney, 0.65 gm.    No. of glomeruli in kidney, 10,123

	Period 1, 12 30 3 22 p m, 2 hrs 52 min	Period 2, 3 22 3 52 p m, 30 min	Period 3, 3 52-4 22 p m, 30 min	Period 4, 4 22 4 42 p m, 20 min	Period 5, 4 42-5 12 p m, 30 min
Volume of urine, cc.	0 57	0 09	0 1	0 12	0 2
"    "    "    "    per hr.	0 199	0 18	0 2	0 36	0 4
Urine uric acid concentration, mg per cent	100 2	120 5	164 5	126 3	121 2
Plasma clearance per hr, cc	1 40	1 53	2 3	3 2	3 4
Blood flow, cc per hr	21 3 (5)	13 5 (7)	16 2 (7)	21 0 (4)	19 7 (7)
Fraction of plasma filtered, per cent	11 7	20 2	25 4	27 2	30 8
Filtrate per glomerulus per hr, c mm					0 34

The figures in parentheses represent the number of determinations

The calculated rate of filtration per glomerulus per hour required to eliminate by filtration all of the uric acid excreted in Period 5 (0.34 c.mm.) is almost exactly equal to that which can be calculated from the data of Experiment 2 in Table VI (0.32 c.mm.) and also to the actual rate of collection of glomerular urine in the same experiment (0.30 c.mm. per hour). The calculated fraction of fluid filtered from the plasma is 30.8 per cent. We have no data from which to decide that this is or is not credible. With the reservation implied in the preceding sentence we may say that the

outcome of these considerations indicates that in none of our experiments is it necessary to assume that any process other than that of glomerular filtration was responsible for the separation from the blood of the uric acid which was actually eliminated by the kidney. We are not inclined at present to make this conclusion more general.

*Other Observations Concerning Glomerular Urine from Snakes.*

*Absence of Protein*—Undiluted glomerular urine from Snake 1, tested for protein with trichloroacetic acid in the manner described, gave no reaction. In three other experiments (Experiments 2, 5, and 6) a portion of the first dilution of glomerular urine was used in the tests. These dilutions were 3.47, 5.1, and 5.3 respectively. The results were negative. Ureteral urine, undiluted, from Snake 2 gave no reaction. The approximate limit of sensitiveness of the test as applied is about 1:700 dilution of frog plasma; i.e., about 0.005 per cent of protein.

*Chlorides*—One qualitative test for chloride has been made on a minute sample of glomerular urine collected in an experiment not reported elsewhere in this paper; it was distinctly positive. In two other experiments chloride was determined in blood plasma and ureteral urine.

	Plasma NaCl per cent	Urine NaCl per cent
Jan. 22	0 68	0 14
" 28	0 81	0 08

From these fragmentary data it appears that, as in the frog, ureteral urine contains considerably less chloride than does blood plasma.

#### DISCUSSION

The evidence which has been presented gives no support to the thought that the glomerular structures in the snake are capable of secretory or selective action with respect to the elimination of uric acid. In snakes and frogs alike the concentration of uric acid in blood and glomerular urine is the same. Our analyses do not uphold the belief of those who, having failed to find uric acid in capsular spaces of fixed sections, decided that uric acid does not normally pass through the glomerular membrane; they point to inadequacy of that technique to yield reliable evidence. Lueken's conclusion from frog perfusion experiments is confirmed and his

results are amplified in that quantitative information gained from living animals is supplied. Acceptance of his conclusion on the basis of his evidence alone requires acceptance of the belief (Scheminzky (21)) that fluid reaching the tubules from efferent vessels does not bathe the second section of the tubules and that the third and fourth sections have no secretory capacity for uric acid. Our aim has been to arrive at a conclusion which could be accepted without any assumptions concerning function of the tubule.

The volumes of glomerular urine which have been collected from frogs indicate that the total volume of glomerular filtrate is amply sufficient to contain all of the uric acid excreted. The volumes of glomerular urine which were collected from snakes in four out of six experiments were conspicuously small. We are inclined to believe that this might have been due to caution, possibly excessive, against reflux of fluid from the tubule, to the small diameter of the tips of our collecting pipettes, and to the ease with which such fine points become partially clogged. It seems certain that in some experiments not all of the glomerular urine formed was collected in the pipette. For these reasons it seems justifiable to use the highest observed rate of glomerular urine collection in considering the possible adequacy of glomerular filtration to explain the uric acid excretion of the whole kidney. In Snake 2, plasma clearance of uric acid, calculated by multiplying the volume of fluid actually collected from one glomerulus by the total number of glomeruli, was 2.3 cc. per hour; calculated from the rate of urine excretion and the uric acid concentrations of urine and plasma it was 2.5 cc. per hour. Although the possibility of error involved in the former computation is great, the coincidence of these two values is striking. Whether it is credible that nearly one-third of the water of the plasma which passes through the kidney can be separated by filtration, as indicated in a separate experiment (p. 216) by measurements of renal blood flow, will be a matter of opinion.

Discussion of the question of the secretion of uric acid by the tubule formed no part of the plan of this paper. It seems right, however, to call attention to the inference which can unquestionably be drawn from the experiments with frogs and, with the reservations indicated, from one of the experiments with snakes, that glomerular filtration was sufficient to account for the excre-

tion of uric acid by the kidney. This inference is at variance with the conclusions drawn from the recent experiments by Lueken and by Marshall. Lueken (9) supplied uric acid to the perfused frog kidney via the renal portal vein and found both the amount and concentration in the urine greater than when it was supplied by the aorta. Marshall (22) gave phlorhizin to lizards (*Iguana iguana*, Shaw), calculated the volume of glomerular filtrate from the glucose values of urine and plasma, and found that this was sufficient to contain only some 5 per cent of the uric acid excreted. The inference from our data stated above, together with the many observations which we have made of the vigor of the glomerular circulation, is responsible for a disinclination yet to believe that glomerular separation is a relatively insignificant factor in uric acid excretion in the animals which we have studied.

We make grateful acknowledgment to Professor Cecil K. Drinker, Harvard School of Public Health, for the facilities of his laboratory from February 15 to July 1, 1931; to Dr. Thomas Barbour, Director of the Museum of Comparative Zoology and University Museum, Harvard University; and to Mr. Roger Conant, Educational Director of the Toledo Zoological Society, who helped us to obtain the small snakes which were particularly useful; to our colleagues Dr. Arthur M. Walker for the collections of glomerular urine noted in Tables III and IV, Dr. B. B. Westfall for a number of uric acid analyses by the original Folin method, and Dr. Elizabeth T. Krick for the glomerular counts.

#### SUMMARY

1. All of the steps of Folin's colorimetric method for determining uric acid, including the preparation of protein-free plasma filtrates, can be conducted in glass capillary tubes. The method, therefore, is applicable to fractions of a cubic millimeter of fluid, containing as little as a few millionths of a milligram of uric acid.

2. The average accuracy of the method thus adapted was found by analysis of known solutions to be of the order of 5 per cent.

3. When frog kidneys are perfused via the aorta with solutions containing uric acid, the concentration of uric acid in the glomerular fluid is the same as that of the perfusion fluid.

4. Glomerular urine collected from the renal corpuscles of living



frogs contains uric acid in the same concentration as that of the blood plasma.

5. Computations of plasma clearance of uric acid by glomerular filtration in frogs, based on uric acid concentration ratios, gave values for average rates of filtration per glomerulus which are well within the range of glomerular urine collections actually made. Hence under the conditions of our experiments the volume of the glomerular filtrate was adequate to contain all of the uric acid excreted by the kidney.

6. The kidneys of small snakes can easily be exposed for microscopic study during life. A considerable number of the glomeruli are visible through the ventral surface. Puncture of Bowman's capsule and withdrawal of glomerular urine in quantities sufficient for analysis has been accomplished in six experiments.

7. The concentration of uric acid in the glomerular urine of snakes is the same as that in the blood plasma. There is, therefore, no ground for believing that the snake glomerulus can "secrete" uric acid.

8. In one snake experiment a computation of total volume of glomerular filtrate from the amount of uric acid excreted and the concentration ratio gave a value nearly identical with that obtained by multiplying the rate of collection from one renal corpuscle by the total number of glomeruli. From this it could be argued that the glomerular filtrate in the snake may contain all of the uric acid excreted. One set of satisfactory estimations of renal blood flow in the snake showed that such a volume of glomerular filtrate may require the filtration of 30 per cent of the plasma which flows through the kidney.

9. The number of glomeruli in the kidneys of the snakes studied varied from 1413 to 17,001.

10. Protein was found to be absent from and chloride present in glomerular urine from snakes.

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## QUANTITATIVE STUDIES OF THE COMPOSITION OF GLOMERULAR URINE

### IX. THE CONCENTRATION OF REDUCING SUBSTANCES IN GLOMERULAR URINE FROM FROGS AND NECTURI DETERMINED BY AN ULTRAMICROADAPTATION OF THE METHOD OF SUMNER. OBSERVATIONS ON THE ACTION OF PHLORHIZIN\*

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The first direct demonstration of the presence of reducing substances in glomerular urine was made by Wearn and Richards (1). Their finding in frogs was confirmed for *Necturus* by White and Schmitt (2). In the work now to be described the concentrations of reducing substances in glomerular urine and plasma of both frogs and *Necturi* have been measured quantitatively. The outcome of the work shows that the concentration in glomerular urine is identical with that in plasma. The bearing of this result upon theories of function of the glomerulus is obvious.

#### *Analytical Method*

To be applicable to this problem the analytical method must be suited to the determination of amounts of glucose of the order of 0.0001 mg. in 0.2 c.mm. of fluid (*i.e.* a concentration of 50 mg. in 100 cc.). Because of previous success with the gasometric estimation of urea nitrogen (3) in exceedingly minute amounts of fluid, we first attempted to adapt the gasometric method of Van Slyke and

\* The expenses of this investigation were defrayed in large part from a grant by the Commonwealth Fund. Preliminary reports of these experiments were made before the National Academy of Sciences, April 25, 1932, and before the American Society of Biological Chemists at Philadelphia, April 29, 1932 (*J. Biol. Chem.*, **97**, lxxii (1932)).

Hawkins (4), then to measure the gas evolved by yeast fermentation. Both attempts failed. We, therefore, turned to the possibilities of microcolorimetry. None of the more commonly used methods proved practicable. Either the colors produced were too faint or the technical procedures too involved. Sumner's method (dinitrosalicylic acid) (5) proved to be ideally suited to our requirements: the procedure was simple, the color intense. In this method 1 cc. of sugar solution is mixed with 3 cc. of reagent,<sup>1</sup> and the mixture immersed in boiling water for 5 minutes. The intensity of the mahogany-red color which develops is measured by comparison in a colorimeter with standard solutions similarly prepared.

The manipulative technique for conducting this reaction quantitatively in capillary tubes has been described in Paper VII of this series (6). Under the microscope 1 volume of the solution to be analyzed (a column 1.5 to 3.0 mm. long) is introduced into a capillary tube of 0.35 mm. inner diameter; then a second column, composed of 3 volumes of reagent, is similarly introduced. The portion of the tube which contains the two fluids is broken off and both ends sealed in a flame. After all the tubes containing a series of fluids to be analyzed have been prepared in this fashion, mixture of the fluids in each tube is effected in all simultaneously by centrifugation.

Since the depth of color produced in minute amounts of fluid in a capillary tube is very nearly the same as that produced by larger amounts of the same fluid in a test-tube, the standard glucose solutions were prepared by the macroprocedure (1 cc. of glucose, 3 cc. of reagent). They covered a range of from 10 to 100 mg. per 100 cc. at intervals of 5 mg.

When all of the standards in test-tubes and all of the unknowns in capillary tubes had been prepared and mixed, they were immersed together in boiling water for 5 minutes. After cooling, the capillary tubes were centrifuged again to recover water of condensation, and the color comparisons made. For this, samples were taken from the standard series into capillary tubes known to be of the same diameter as those which contained the unknowns. Comparisons were made against white paper in a room darkened

<sup>1</sup> In addition to the sodium salt of dinitrosalicylic acid, the reagent contains sodium bisulfite, sodium and potassium tartrate, and phenol.

save for the electric lamp used to illuminate the working space. This was screened with Daylite glass.

The color comparisons are decidedly more difficult than is the case with the blue colors produced in determining uric acid or phosphate. But after practise and with due care, it is not difficult to distinguish between the colors of standards which differ by 5 mg. of glucose per 100 cc. When the color of an unknown solution was found to be between those of two standards an intermediate value was assigned to it. In every estimation independent comparisons were made by two observers. These rarely differed by more than 2 mg. per 100 cc., never by more than 5 mg. The accepted result was the average of the two readings.

It was found that when columns of glucose solution shorter than 1.5 mm. were taken the color developed was less intense than normal. It was also found that as the dinitrosalicylic acid reagent aged (1 to 4 weeks) the color developed in a capillary tube sometimes became more intense than that developed in a test-tube by about 5 mg. per 100 cc. over the entire range of our solutions; this discrepancy could be corrected by preparing a fresh lot of reagent, by making the standard solutions by micromethod, or by the introduction of a correction.

#### *Analyses of Known Glucose Solutions*

At frequent intervals during 6 months, duplicate analyses were made upon known glucose solutions. In every instance one or both of the persons taking part in the analysis were ignorant of the true concentration, until the determination was completed. In Table I are reported all such results obtained during 3 consecutive weeks. The average error of duplicate determinations only once exceeded 3 mg. per 100 cc.; in only one of the forty-six separate determinations did the error exceed 4 mg. per 100 cc. The mean error of the series was +1.1 mg. per 100 cc., the average deviation from the mean, 1.5. We conclude, therefore, that the analytical error of the method does not exceed 4 mg. per 100 cc.

#### *Analyses of Frog Plasma*

Protein in the concentration in which it exists in frog plasma does not interfere with the accuracy of the method as described

above. Deproteinization was therefore unnecessary. Two series of control tests were made.

The fermentable reducing substances in six specimens of frog plasma were removed by treatment with washed yeast (7). Micro-analyses of these plasmas indicated the presence of less than 10 mg. per 100 cc. of non-fermentable reducing substances. The colors were too pale to permit accurate estimation. The concentration was judged to be not far from 5 mg. per 100 cc.

TABLE I

*Determinations of Glucose in Solutions of Known Concentration*

The results are expressed as mg. of glucose per 100 cc.

Experiment No	Concentration of solution			Error	Experiment No	Concentration of solution			Error
	Found		Known			Found		Known	
	Duplicates	Average				Duplicates	Average		
1	28, 30	29	30	-1	13	53, 55	54	54	0
2	31, 33	32	30	+2	14	60, 60	60	60	0
3	29, 33	31	30	+1	15	60, 61	61	61	0
4	31, 33	32	31	+1	16	71, 71	71	70	+1
5	32, 35	34	31	+3	17	71, 74	73	70	+3
6	41, 44	43	37	+6	18	69, 71	70	72	-2
7	37, 40	39	38	+1	19	73, 75	74	72	+2
8	35, 37	36	38	-2	20	71, 73	72	73	-1
9	48, 49	49	46	+3	21	83, 81	82	84	-2
10	47, 49	48	46	+2	22	95, 97	96	94	+2
11	49, 51	50	47	+3	23	100, 100	100	99	+1
12	57, 58	58	55	+3					
Mean									+1 1
“ deviation									±1 5

To each specimen, pure anhydrous glucose (Pfanstiehl) was added in known amount by a person not concerned in the investigation. Analysis was made as described above, with a 2.5 mm. column of plasma in the capillary tube. The results are shown in Table II. They indicate that the glucose found exceeded the glucose added by from 1.5 to 7.0 mg. per 100 cc. (average 4.6). The discrepancy can be attributed to the non-fermentable reducing substances and we conclude that the method is accurate for the determination of glucose added to plasma.

In eight experiments comparison was made between our micro-adaptation of Sumner's method and the Hagedorn-Jensen method (8) carried out by macromethod. We are indebted to Dr. B. B. Westfall of this laboratory for the Hagedorn-Jensen estimations. They were made on zinc hydroxide filtrates from plasma. Filtration was made by suction through No. 4 Whatman paper less than 2 cm. in diameter. The results obtained by the two methods are grouped in Table II. In no case did they differ by more than 4 mg. per 100 cc.; the mean difference was  $-0.8$  mg. per 100 cc.

TABLE II

*Determinations of Reducing Substances in Frog Plasma*

The results are expressed as mg. of glucose per 100 cc. of plasma.

Recovery of glucose added to glucose-free plasma				Comparison of analyses by Hagedorn-Jensen and ultramicromethods			
Experiment No	Glucose added	Glucose found*	Difference	Experiment No	Hagedorn*	Ultramicro-method*	Difference
1	32 6	38 0	+5 4	1	37 9	38 0	+0 1
2	41 6	48 0	+6 4	2	39 9	38 0	-1 9
3	45 0	52 0	+7 0	3	44 5	46 0	+1 5
4	50 0	55 0	+5 0	4	47 5	48 0	+0 5
5	62 5	65 0	+2 5	5	58 8	56 0	-2 8
6	82 5	84 0	+1 5	6	64 4	62 0	-2 4
				7	69 3	72 0	+2 7
				8	80 0	76 0	-4 0
Mean	..		+4 6†				-0 8

\* Each figure presented is the average of two or more determinations.

† The fermented plasmas were shown to contain approximately 5 mg. per 100 cc. of saccharoids.

We conclude that the substances in plasma estimated by the Sumner method are the same as those measured by the Hagedorn-Jensen method and that our adaptation is as reliable when applied to frog plasma as when applied to pure watery solutions.

*Filtrability of Reducing Substances of Plasma*

Twelve experiments were made in which frog plasma was filtered through cellophane at pressures of about 200 mm. of Hg. The unfiltered plasma and the protein-free filtrate were analyzed in duplicate by our micromethod. The reducing substance concentration of the plasma varied from 38 to 68 mg. per 100 cc.



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The following figures show the differences between ultrafiltrate and plasma, expressed as mg. of glucose per 100 cc.

+1, -2, +1, +1, +5, +2, +4, -4, -4, -3, -1, 0      Mean, 0

Two experiments with similar results were made with sterile normal horse serum diluted with 2 parts of Ringer's solution.

We conclude that all the reducing substances of frog plasma are filtrable through cellophane.

### *Perfusion Experiments on Frog Kidney*

In sixteen experiments the kidneys of *Rana pipiens* were perfused via the aorta with artificial solutions to which glucose in known amount had been added; glomerular fluid was collected, and its sugar content and that of the perfusion fluid were determined by microanalysis. In five experiments the perfusing fluid was oxygenated Ringer's solution, containing 0.02 to 0.1 per cent of sodium bicarbonate; in eleven, sterile normal horse serum diluted with 1 or 2 volumes of Ringer's solution, and the fluid then oxygenated. Anhydrous glucose was added to yield concentrations of from 0.04 to 0.08 per cent, and, in eight experiments, the urea concentration of the perfusing fluid was increased by 20 mg. per 100 cc. There was usually no interruption in the renal circulation before beginning the perfusion. Perfusion pressures varied between 20 and 50 cm. of H<sub>2</sub>O. In three experiments bladder urine was analyzed; the results showed that the kidneys retained their power to reabsorb glucose. Details of glomerular fluid collection were similar to those described below in connection with the experiments on living frogs.

Microanalyses were made in duplicate upon two specimens of perfusion fluid in each experiment. The results are collected in Table III. In eleven experiments the determined difference between the glucose concentrations of glomerular fluid and perfusion fluid was equal to or less than 4 mg. per 100 cc., *i.e.*, the figure accepted as maximum analytical error. In four of the remaining five experiments the difference was equal to or less than 8 mg. per 100 cc., *i.e.* twice the possible error of a single determination. We conclude that the sugar content of glomerular fluid in perfused frog kidneys is the same as that of the perfusion fluid.

TABLE III

*Experiments in Which Frog Kidneys Were Perfused with Saline or Diluted Horse Serum*

The results are expressed as mg. of glucose per 100 cc.

Experiment No.	Perfusion fluid	Perfusion fluid*	Glomerular urine†	Difference	Notes
1	Ringer's solution	65	63	-2	
2	" "	81	85	+4	
3	" "	77	77	0	Same frog as in Experiment 2, different glomerulus
4	" "	47	48	+1	
5	" "	47	48	+1	Capsule torn. Same frog as in Experiment 4, different glomerulus
6	50 per cent horse serum	63	66	+3	
7	50 " " " "	50	45	-5	
8	33 " " " "	71	68	-3	
9	33 " " " "	47	47	0	
10	33 " " " "	47	41	-6	
11	33 " " " "	82	92	+10	Tubule not blocked
12	33 " " " "	64	60	-4	Protein in glomerular urine
13	33 " " " "	58	57	-1	
14	33 " " " "	59	52	-7	
15	33 " " " "	59	51	-8	Same frog as in Experiment 14, different glomerulus
16	33 " " " "	79	78	-1	
Mean.				-1	1
" deviation				±3	4

\* Average of four determinations.

† Average of two determinations in Experiments 3, 7, and 12; elsewhere single determination.

### *Experiments on Living Frogs*

Thirty-four experiments have been made on healthy female *Rana pipiens*: thirty-two between September 23 and December 3, 1931, and two in May, 1932. One important addition to operative technique was required because of the change in concentration

of blood sugar which occurs after destruction of the brain. In the succeeding hour, the blood sugar may rise as much as 50 mg. per 100 cc. and the rise continues for at least another hour. A similar rise follows the use of urethane, amytal, or even prolonged restraint. It is not obviated by artificial respiration. Hence, in each experiment, soon after the brain was crushed with a hemostat, the circulation through the liver was abolished by ligation of the celiaco-mesenteric artery and the portal vein, and that through the muscles restricted by a mass ligature about each thigh. By these procedures the rise in blood sugar was materially diminished.

No saline was injected into any of the frogs excepting the seven whose glomerular urine was to be analyzed for uric acid; these were given a subcutaneous injection of 2 cc. of 0.1 per cent lithium urate 30 minutes before they were pithed.

The right kidney was exposed and illuminated by transmitted light. Glomerular urine was collected in a quartz capillary pipette in the usual way. The tubule from the renal corpuscle chosen for puncture was identified by intracapsular injection of phenol red and blocked by pressure with a glass rod after the tip of the pipette and the capsular space had been washed free of dye by freshly formed glomerular urine. In twenty-seven instances the neck of the tubule was the part compressed, in seven a portion of the proximal convolution. Assurance of completeness of obstruction was often gained from the fact that dye could be seen to remain stationary in a part of the tubule distal to the point of compression during the period of glomerular urine collection.

As a further precaution against reflux of fluid from the tubule, the mercury bulb of the pipette system was held at least 20 mm. above the surface of the kidney for the first few minutes; then it was gradually lowered to the final level recorded in Table IV (usually 3 to 10 mm. above the kidney). In three experiments contact of glomerular urine with mercury was prevented by introducing a column of oil into the tip of the pipette. In seven experiments 2 to 4 drops of 2 per cent caffeine were placed on the surface of the kidney during collection.

All of the preliminary operations before the glomerular urine collection was begun were usually completed in less than an hour. The average time of collection was 54 minutes. The average amount of glomerular urine obtained was 0.48 c.mm.

During each collection of glomerular urine two samples of blood (about 0.015 cc. each) were taken from the ventricle of the heart into capillary pipettes containing a few grains of dry powdered potassium oxalate;<sup>2</sup> the first within the first 10 minutes, the second during the last 10 minutes of the collection. The plasma was immediately separated by centrifugation.

At the end of a glomerular urine collection, the fluid was transferred to the capillary tube in which the color reaction was to be conducted. Transfer was usually completed within 3 minutes. When the amount of fluid sufficed for more than a single determination, succeeding transfers were made at intervals of about 5 minutes.

Details of the microanalytical procedures have been described above.

In every experiment tests for the presence of protein in glomerular urine were made with trichloroacetic acid as described elsewhere (6). In fourteen experiments, undiluted glomerular urine was used: in five no protein was detected; in nine it was present in concentration judged to range from 0.008 to 0.035 per cent, if frog plasma is assumed to contain 3.5 per cent of protein. In twenty experiments the test was made upon the sugar reaction mixture after the colorimetric estimation had been finished. A positive result was obtained in four of these. The failure to detect protein in the rest does not signify absence of protein from the original fluid because of dilution by the reagent and destruction of protein by the alkali in it. It was obvious that in a considerable number of animals the glomerular membrane was pervious to a small fraction of the plasma protein.

*Phlorhizin*—In five experiments (Experiments 21 to 24 and Experiment 29) phlorhizin glycosuria was established before the glomerular urine was collected. Two frogs received each a single dose of 2 mg. of phlorhizin 2 hours before the experiment; one received six 2 mg. doses, and two received seven 20 mg. doses during 48 hours; the last dose was given 2 hours before collecting bladder urine and beginning the experiment.<sup>3</sup>

<sup>2</sup> The use of heparin is not permissible.

<sup>3</sup> A 1 per cent solution of phlorhizin in 1.2 per cent sodium carbonate produces a barely detectable color when heated with the dinitrosalicylic acid reagent.



18	+70.19	51	39, 40	42, 43	41	41	0	Uric acid and total molecular concentration also determined. Greater part of glomerular fluid collected near end
19	+20.79	85	47, 49	63, 67	57	65	+8	
20	-20.72	84	45, 46	57, 59	52	52	0	Uric acid and total molecular concentration also determined
25	+50.19	30	69, 70	54, 57	66†	63	-3	Artificial respiration. Capsule probably torn
26	+10.36	65	90, 92	98, 100	95	78, 82	-15	"
27	+50.76	39	48, 52	57, 59	54	51, 52	-2	Uric acid also determined. Artificial respiration
28-A	+70.94	36	46, 48	52	50	45, 48	-3	"
30	+10.39	56	30, 32	33	32	33, 33	+1	Artificial respiration
31	+70.37	55	55, 57	67, 68	62	61	-1	"
32	+70.31	37	18, 18	18, 19	18	18	0	" Hemorrhage before experiment
33	+80.79	31	52, 53	55, 56	54	46, 48	-7	Artificial respiration
21	-40.0	50	66, 87, 88	94, 97	92	88, 92	-2	2 mg. phlorhizin. Bladder urine 160 mg. per cent
22	-50.55	43	87, 87	96, 98	92	86, 88	-5	" " " 175 " " "
23	+100.28	28	101, 103	104, 104	103	97	-6	12 " " " 55 " " "
24	+40.31	57	78, 78	78, 78	78	68	-10	Artificial respiration 140 mg. phlorhizin. Bladder urine 85 mg. per cent. Artificial respiration. Cardiac hemorrhage during collection
29	+50.24	40	41, 43	42, 43	42	38	-4	140 mg. phlorhizin. Bladder urine 33 mg. per cent. Artificial respiration. Tubule block probably not complete
Mean...	0.48	54			59.1	57.1	-2.2	
Mean deviation.....							±3.0	

\* Recorded volume not always accurate, a portion of the fluid being occasionally lost during its transfer.

† Plasma 2 was taken after glomerular urine collection was finished; "average" value is therefore interpolated.

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*Experiments Omitted*—Twelve experiments have been excluded from consideration in this report. In two instances the tubule was not blocked; in three the plasma sugar concentration rose more than 20 mg. per 100 cc. during the course of the glomerular urine collection; in three hemoglobin in the plasma interfered with the colorimetry; in three the volume of glomerular fluid was too small for reliable analysis; in one the glomerular fluid contained a large amount of protein.

### *Experiments on Necturus maculosus*

Between April 27 and May 4, 1932, four specimens of glomerular urine were collected from healthy *Necturi*. Two of the collections were made in this laboratory by Dr. H. L. White of St. Louis, the other two by one of the authors. Anesthesia was induced by immersion in 1.5 per cent urethane for 5 to 10 minutes and maintained during the operation by submerging the upper portion of the body and the gills in 0.15 per cent urethane. Blood was taken from the posterior cava by a finely pointed pipette shortly before the beginning and soon after the end of a glomerular urine collection. Circulation through the glomerular tufts was active. Collection of glomerular urine was made against pressure sufficient to prevent its contamination by fluid entering the tubules through the nephrostomes. No attempt was made to block the tubules nor to lessen the rise in blood sugar which occurred during each experiment. In no instance was sufficient glomerular urine obtained to permit the sugar analysis to be made in duplicate or to test for protein.

### *Results*

*Frogs*—The essential details and the results are grouped in Table IV. The correspondence between the values for the reducing power of glomerular urine and plasma is striking. This correspondence persisted at plasma values that ranged from 18 to 103 mg. per 100 cc. In twenty-three experiments the difference was only 0 to 4 mg. per 100 cc.; in nine it was between 4 and 8; in only two did it exceed this. Since each "difference" in Table IV is obtained from at least four separate estimations, each with its own error (p. 225), it appears that the value of the reducing power of glomerular urine is the same as that of plasma within the errors

of the analytical method used. It will be noted, however, that in only six instances is the figure for glomerular urine higher than that for plasma; in twenty-two it is less; and the calculations for plasma have not been made on the basis of its water content.

TABLE V

*Reducing Substances in Venous Blood Plasma and Glomerular Urine from Necturi*

The results are expressed as mg. of glucose per 100 cc.

Experiment No.	Plasma 1	Plasma 2	Average	Glomerular urine	Difference
1	32*	36, 37	34	34	0
2	48*	56, 56	52	56	+4
3	57, 57	73, 73	65	67	+2
4	37, 37	53, 54	45	49	+4

\* Plasma was not collected immediately before the experiment; recorded figure obtained by interpolation.

TABLE VI

*Analysis of Frog Plasma and Glomerular Urine for More Than One Constituent*

Experiment No.	Uric acid*			Reducing substances			Total molecular concentration†
	Plasma	Glomerular urine	Difference	Plasma	Glomerular urine	Difference	Glomerular urine
	mg per 100 cc.	mg per 100 cc.	per cent	mg. per 100 cc.	mg per 100 cc.	per cent	per cent
1	8 0	7 2	-10	37	35	-5	
2	3 2	3 6	+13	57	57	0	
3	3 2	3 2	0	73	74	+1	
4	4 7	4 9	+4	57	65	+14	+0 2
5	4 3	4 3	0	52	52	0	-0 1
6	8 8	9 4	+7	54	52	-4	
7	5.4	5 6	+4	50	47	-6	

\* Analyses by Dr. James Bordley, 3rd.

† Analyses by Dr. Elizabeth Krick. The figures represent percentage change in the length of the column of glomerular urine.

These figures make it seem possible that a small fraction of the sugar dissolved in the water of the arterial blood is lost before it is separated as glomerular urine. It may be that there is utilization of sugar by the cells of the glomerular membrane or by the



structures of the walls of the minute vessels through which the blood flows before it reaches this.<sup>4</sup>

The average concentration of reducing substances in twelve specimens of bladder urine formed during the course of an experiment was 13 mg. per 100 cc.; that of the glomerular urine 57 mg. These results confirm the tubular reabsorption of sugar which was proved by Wearn and Richards (1).

*Necturi*—The comparisons of reducing power of venous blood plasma and glomerular urine shown in Table V indicate identity. All of the observed differences are well within the limits of analytical error.

*Phlorhizin*—In none of the experiments in frogs in which phlorhizin was injected did the reducing power of glomerular urine exceed that of the plasma. Analyses of bladder urine show that the typical action of this substance had developed. The conclusion is inescapable, therefore, that increased glomerular elimination of sugar is not a factor in phlorhizin glycosuria in frogs.

*Other Estimations*—In Table VI data are presented from experiments in which enough glomerular urine was obtained to permit determination of more than one constituent. The figures for glucose are taken from Table IV of this paper, for uric acid from Table IV of Paper VIII of this series (9). The agreement of the several differences between glomerular urine and plasma is such as to give added confidence in the reliability of the analytical results.

#### CONCLUSIONS

The dinitrosalicylic acid method of Sumner for the determination of reducing substances in urine has been so adapted to use in small glass capillary tubes that it is possible to perform analyses on 0.2 c.mm. of fluid. The maximum error in duplicate determinations of known glucose solutions is in the neighborhood of 3 mg. per 100 cc. The results on frog plasma are almost identical with those obtained by the Hagedorn-Jensen method. Ultrafiltration experiments indicate that all of the reducing substances in frog plasma are filtrable through cellophane membranes.

<sup>4</sup> The concentration of reducing substances in venous plasma taken from the inferior vena cava averaged (five experiments) 7 mg. per 100 cc. less than that of arterial plasma simultaneously collected by cardiac puncture.

Thirty-eight experiments on frogs and *Necturi* have been completed in which the concentrations of reducing substance in glomerular urine and plasma have been compared. The mean difference between the two is only  $-1.7$  mg. per 100 cc. This difference is judged to lie within the experimental error of the method employed and it is, therefore, concluded that the reducing substance content of the two fluids is identical. Such a conclusion extends the previous evidence from this laboratory which indicates that the function of the glomerulus is one of filtration.

In 1924, Wearn and Richards demonstrated that the tubules of the frog kidney reabsorb glucose. This demonstration is confirmed.

Phlorhizinized frogs do not differ from normal frogs with respect to the comparative reducing power of glomerular urine and plasma. Phlorhizin glycosuria cannot therefore be attributed to alteration in glomerular elimination. Inasmuch as the urine to plasma ratio of reducing substances did not exceed 2.0, no reason is apparent for ascribing the glycosuria to any change other than decrease in reabsorption from the tubule.

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# QUANTITATIVE STUDIES OF THE COMPOSITION OF GLOMERULAR URINE

## X. THE CONCENTRATION OF INORGANIC PHOSPHATE IN GLOMERULAR URINE FROM FROGS AND NECTURI DETERMINED BY AN ULTRAMICROMODIFICA- TION OF THE BELL-DOISY METHOD\*

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In view of existing evidence that inorganic phosphate of serum is capable of passing through membranes impermeable to protein, and in view of the increasing volume of evidence that glomerular urine has the composition of an ultrafiltrate, it is difficult to believe that part at least of the inorganic phosphate of urine is not excreted through the glomeruli. In compensation dialysis experiments, Rona and Takahashi (1) found phosphate of horse serum to be completely diffusible. Cushny (2) found 3.9 mg. per cent of phosphate phosphorus in an ultrafiltrate of ox serum. Neuhausen and Pincus (3) filtered pig serum through collodion and found slightly higher values of inorganic phosphate in the filtrate than in the serum. Grollman (4) found that 100 per cent of the inorganic phosphate in normal pig and dog serum was filtrable; in frog serum, 85 per cent. He was able to decrease the filtrability of phosphate by the addition of calcium chloride to the serum.

That the fluid separated from the blood in passing through the glomerulus has the composition of an ultrafiltrate is shown by the

\* The expenses of this investigation were defrayed in large part from a grant by the Commonwealth Fund. Preliminary reports of these experiments were made before the National Academy of Sciences, April 25, 1932, and before the American Society of Biological Chemists at Philadelphia, April 29, 1932 (*J. Biol. Chem.*, **97**, lxxii (1932)).

series of papers from this laboratory (5-9), of which this is one, and by a mass of less direct evidence.

On the other hand, Starling and his collaborators and Schmitt and White (10) have published evidence which indicates that glomerular filtration does not account for the inorganic phosphate of bladder urine. Eicholtz and Starling (11), perfusing dog kidney in heart-lung-kidney experiments, found no phosphate in the urine; the addition of sodium phosphate to the perfusing blood was followed by only transitory excretion of this substance. The previous discovery by Robison (12) of a phosphoric esterase in kidney tissue, together with the finding of Eicholtz, Robison, and Brull (13) that the addition of glycerophosphate to the blood with which kidneys were perfused led to the urinary excretion of inorganic phosphate, gave rise to the view that phosphates normally exist in the blood in a combination incapable of passing through the glomerular membrane and that the phosphates normally present in urine are the result of secretory activity of the renal tubule in which phosphoric esterase plays a part. Schmitt and White, in experiments upon *Necturi*, found only one-quarter to one-third as much phosphate in glomerular urine as in blood plasma.

This conflict of evidence could, we believed, be partially settled by more thorough analytical study than had yet been made of the phosphate content of glomerular urine. The work reported in this paper represents an effort in this direction. In it the filtrability of inorganic phosphates in the plasma of frogs and *Necturi* has been demonstrated; Kuttner's modification of the Bell-Doisy method for determination of phosphates has been satisfactorily adapted to the analysis of minute amounts of fluid; and the concentration of inorganic phosphates in glomerular urine of frogs and *Necturi* has been found to be approximately the same as that of the plasma. Belief in the glomerular filtration of phosphates is therefore supported.

#### *Analytical Method*

In Kuttner's modification (14, 15) of the Bell-Doisy method (16), the reduction of phosphomolybdic acid is accomplished by stannous chloride rather than by hydroquinone. The blue color which results is more intense; it develops immediately and the reaction does not require heat. In these respects the method is

superior for our purpose to that of Benedict and Theis which was similarly adapted to small amounts of fluid by Schmitt and White (10). When the reaction is conducted in small capillary tubes, the color developed from concentrations of phosphate such as are present in blood is sufficiently intense for satisfactory quantitative comparison with standards. By the capillary tube colorimetric technique described by Richards, Bordley, and Walker (17), we have analyzed as little as 0.08 c.mm. of fluid having a phosphate content of less than 0.000001 mg. with accuracy scarcely less than that of the macromethod.

The proportions in which Kuttner's reagents are mixed with the fluid to be analyzed differ in this ultramicroadaptation from those originally recommended by him. This change, adopted in order to lessen the dilution of glomerular fluid or plasma, involved an increase in the concentration of stannous chloride and a slight reduction in acidity of the final mixture. The stock solution of 40 per cent stannous chloride was freshly prepared each week; the 1:100 dilution, daily.

*Procedure*—1 volume each (about 0.2 c.mm.) of the fluid to be analyzed and of the molybdic-sulfuric acid reagent was introduced into a glass capillary tube (0.35 mm. inner diameter) by the method which has been described (17). The two fluid columns were withdrawn 3 cm. from the end of the capillary and an equal volume of the diluted stannous chloride solution was taken in. The portion of the tube containing them was broken off, its ends sealed in a minute gas flame, and laid aside in a horizontal position while a series of capillaries containing the standard phosphate solutions was similarly prepared. Between concentrations of 1.5 and 7.0 mg. of phosphorus per 100 cc. these standard solutions differed from each other by 0.5 mg.; below 1.5 mg., by 0.1 or 0.2 mg. Since the approximate value of the unknown solution had been found by preliminary determination, it was usually unnecessary to prepare more than four standard solutions in one series. The tubes were placed together in the centrifuge with the stannous chloride column uppermost, and the fluids within the capillaries mixed simultaneously by centrifugation (17). The blue color developed immediately and completely. The capillaries were placed on a sheet of unglazed milk glass, so illuminated by two electric lights as to avoid shadows, and the readings made by comparing the color of

the unknown solution with those of the standard solutions (17). There was never the slightest difficulty in distinguishing one standard solution from another. The colors faded by about 10 per cent during the first few minutes after centrifugation, but since this fading occurs equally in all capillaries of a single series, the readings could be made at any time within 2 hours. It was our practise to make them immediately. Independent readings by two observers were made on each capillary. Determinations were usually made in duplicate, each with its own set of standards. In the analysis of bladder urine, in which the concentration was often greater than 7.0 mg. per 100 cc., a preliminary dilution with 1 or more volumes of water was required.

If any considerable amount of protein is present in the fluid to be analyzed, the blue color is unevenly distributed after centrifugation. In determinations upon lymph or plasma, it was therefore necessary to use protein-free filtrates. Such filtrates have been obtained from small amounts of plasma<sup>1</sup> in capillary tubes by adding one-fortieth of its volume of 90 per cent (by weight) trichloroacetic acid and repeatedly centrifuging the mixture (17). The "filtrate" obtained in this way from 0.01 cc. of frog or *Necturus* plasma is perfectly clear, is sufficient in amount for a dozen analyses, and does not contain enough protein to interfere with the colorimetry. Blood was collected in a glass capillary pipette by cardiac puncture or venipuncture, the plasma separated by centrifugation, and the cells discarded by cutting the tube.

*Analyses of Known Phosphate Solutions*—A typical series of consecutive analyses of aqueous phosphate solutions is summarized in Table I. The values of these solutions were unknown to one or both of us until the analysis was completed. When one of us knew the true value of the solution, the identity of the capillaries was concealed during the colorimetry. Duplicate determinations were made on each solution and the average recorded as the accepted result. In only two instances was the error greater than 5 per cent; the mean error was +0.1 per cent, the average deviation from the mean,  $\pm 2.5$ .

<sup>1</sup> An excess of potassium oxalate produced a marked diminution in color. Several of our early experiments were unacceptable on this account. No anticoagulant is necessary with frog or *Necturus* blood if the procedures leading to deproteinization are rapidly executed.

The Kuttner method is said (15) to be specific for inorganic phosphates. The only organic phosphate which we have tried (calcium glycerophosphate) produced no apparent coloration in a concentration of 0.1 per cent.

*Analyses of Plasma*.—Nine specimens of frog plasma and two of horse serum, diluted with 1 volume of 0.9 per cent sodium chloride, were analyzed both by our micromethod and by the macro-method of Kuttner. A majority of the macrodeterminations was made for us by Dr. B. B. Westfall in this laboratory. The results of the two methods are compared in the first part of Table

TABLE I  
*Determinations of Inorganic Phosphate in Solutions of Known Concentration*

Experiment No	Concentration of solution, P per 100 cc		Error	Experiment No	Concentration of solution, P per 100 cc		Error
	Found*	Known			Found*	Known	
	mg	mg	per cent		mg	mg	per cent
1	0 85	0 8	+6.3	8	4 6	4 6	0 0
2	1 35	1 4	-3 6	9	4 8	4 8	0 0
3	1 75	1 8	-2 8	10	5 4	5 3	+1 9
4	2 50	2 5	0 0	11	5 7	5 7	0 0
5	2 50	2 7	-7 4	12	6 3	6 1	+3 3
6	3 15	3 2	-1 6	13	6 7	6 6	+1 5
7	3 75	3 6	+4 2				
Mean error							+0 1
" deviation .							±2 5

\* Average of duplicate determinations.

II; the difference was greater than 5.5 per cent only once and the mean difference was -1.5 per cent.

In a second series frog plasma was dialyzed against 0.6 per cent sodium chloride until a protein-free filtrate from it gave no phosphate reaction.<sup>2</sup> It was divided into seven portions and known phosphate solution (unknown to us) added in different amount to each. The phosphate content of each was determined

<sup>2</sup> If the plasma was not deproteinized, a definite blue color appeared when the reagents were added. We were unable to estimate the depth of this color because it was unequally distributed in the capillary.



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in duplicate by the micromethod after deproteinization. The results are summarized in the second part of Table II. In each

TABLE II  
*Determinations of Inorganic Phosphate in Plasma*

Experiment No (1)	Source (2)	P per 100 cc by Kuttner method* (3)	P per 100 cc by micromethod* (4)	Difference (4) - (3)
		<i>mg</i>	<i>mg</i>	<i>per cent</i>
1	Horse	2 64	2 5	-5 3
2	"	2 67	2 8	+4 9
3	Frog	1 95	1 8	-7 7
4	"	2 10	2 1	0 0
5	"	3 54	3 4	-4 0
6	"	3 60	3 4	-5 5
7	"	4 15	4 15	0 0
8	"	4 22	4 4	+4 3
9	"	4 59	4 5	-2 0
10	"	5 02	5 1	+1 6
11	"	6 66	6 5	-2 4
Mean difference				-1 5
" deviation . .				±3 3

## Recovery of phosphate added to

Phosphate-free frog plasma				Phosphate-containing frog plasma			
Experiment No	Concentration of P per 100 cc		Difference	Experiment No	Concentration of P per 100 cc		Difference
	Added	Found*			Added	Found*	
	<i>mg</i>	<i>mg</i>	<i>per cent</i>		<i>mg</i>	<i>mg</i>	<i>per cent</i>
1	2 48	2 3	-7 2	1	2 69	2 6	-3 3
2	3 21	3 05	-5 0				
3	3 55	3 3	-7 0	2	2 92	2 9	-0 7
4	3 92	3 55	-9 4				
5	4 14	3 9	-5 8	3	3 58	3 6	+0 6
6	4 94	4 6	-6 9				
7	5 21	4 95	-5 0	4	3 94	3 9	-1 0
Mean difference			-6 6				-1 1

\* Average of duplicate determinations

instance the phosphate found was from 5.0 to 9.4 per cent less than that added.

To learn whether this difference was caused by a defect in method, we collected another specimen of frog plasma and carefully determined its phosphate concentration by our method. It was divided into four portions and known phosphate solution added to each. The results of analysis of these reinforced plasmas showed that 99 per cent of the added phosphate had been determined. We have not been able to find previous reports of de-

TABLE III

*Phosphate Determinations on Ultrafiltrates (Cellophane) of Various Bloods*

Source (1)	P per 100 cc serum* (2)	P per 100 cc ultrafiltrate* (3)	Difference (3) - (2)
	<i>mg</i>	<i>mg</i>	<i>per cent</i>
Frog†	2 9	3.0	+3 4
"	3 0	3 2	+6 7
"	4 6	4 6	0 0
"	4 7	4 45	-5 3
"	5 5	5 65	+2 7
"	6 5	6 35	-2 3
<i>Necturus</i>	3 0	3 1	+3 3
"	3 0	3 1	+3 3
"	3 6	3 7	+2 8
"	4 9	5 1	+4 1
Horse	2 4	2 4	0 0
"	2 7	2 7	0 0
Human	3 1	3 2	+3 2
"	3 9	3 8	-2 6
"	4 1	4 2	+2 4
Mean difference			+1 4
" deviation			±2 5

\* Average of duplicate determinations.

† In this instance a parlodion sac was used.

terminations of phosphate added to phosphate-free plasma. The results given above indicate that from 5 to 9 per cent of the inorganic phosphate in frog plasma escapes determination both in Kuttner's macromethod and in our microadaptation of it. It may be presumed that this fraction exists in such a state as to be carried down with the protein precipitate.

*Filtrability of Plasma Phosphate Through Artificial Membranes*—The only determination of phosphate in an ultrafiltrate of frog

plasma of which we are aware is the single estimation made by Grollman (4). He found that 85 per cent was filtrable. No figures for ultrafiltrates from *Necturus* or human serum were discovered. Additional data seemed desirable. We have, accordingly, performed the fifteen experiments summarized in Table III. About 0.1 cc. of serum was placed in a glass filtering tube, one end of which was closed with a cellophane membrane. Evaporation was prevented by covering the serum with oil and immersing the membrane in oil. Filtration pressure was 200 mm. of mercury. In only one of the fifteen instances was the difference between the phosphate concentration of the filtrate and that of the serum greater than 5.5 per cent; the mean difference was +1.4 per cent. We conclude that all of the inorganic phosphate in plasma or serum from the frog, *Necturus*, horse, and man which can be determined in a trichloroacetic acid filtrate is filtrable through a cellophane membrane.

*Phosphate Content of Lymph*—In six instances lymph was obtained from the web of the frog's foot and its phosphate content compared with that of blood plasma (18). The mean value of the lymph in these experiments was 94 per cent that of the plasma. We are informed by Dr. Walter Bauer that a similar relationship exists between the phosphate contents of blood and synovial fluid from cattle.

#### *Phosphate Concentration of Glomerular Urine in Frogs*

*Procedure*—The experiments were performed between February 17 and April 22, 1932, on fifteen healthy female *Rana pipiens*. A subcutaneous injection of about 2.0 cc. of 0.6 per cent sodium chloride was routinely given as a preliminary to operation, but thereafter no fluid of any sort was given subcutaneously or intravenously. In thirteen animals artificial respiration was continued throughout the operation and experiment. The right kidney was exposed for direct observation and the collection of glomerular urine from one of its glomeruli usually started within 40 minutes of the time the animal was pithed. The average duration of the collection was 45 minutes; the average amount of fluid collected, 0.51 c.mm. In eight instances this amount was sufficient to permit duplicate or triplicate phosphate analyses; in the re-

maining instances only a single analysis was made. In ten instances there was sufficient fluid to allow a protein test with 20 per cent trichloroacetic acid upon an undiluted specimen (17): six of these specimens were negative; in four the protein reaction was comparable to that obtained with frog plasma diluted from 100 to 500 times. Contamination of glomerular fluid by tubule contents was prevented by maintaining a positive intracapsular pressure and by compressing the neck of the tubule; obstruction of the tubule lumen was considered satisfactory in fourteen experiments and in the other two we believed that no contamination occurred. In three experiments, 1 or 2 drops of 2 per cent caffeine were dropped upon the kidney surface during the collection.

Within 5 minutes of the time the glomerular urine collection was started, about 0.02 cc. of blood was obtained by cardiac puncture. Its plasma was separated, deproteinized, and the approximate phosphate concentration estimated by a preliminary determination. About 5 minutes before the end of the collection a second specimen of cardiac blood was secured and its plasma deproteinized. When sufficient glomerular urine had been secured, the pipette was withdrawn from the capsule and a portion or all of its contents transferred to a capillary tube (17). The preparation of this capillary was usually completed within 4 minutes of the end of the experiment. Capillaries containing portions of the two plasma specimens and standard phosphate solutions covering the anticipated range were then prepared. After the colorimetric readings on this series were completed, a second series of capillaries was prepared containing duplicates of the two plasmas, standard phosphate solutions, and a second portion of the glomerular fluid, if that were available. The plasma phosphate level never changed more than 0.4 mg. per 100 cc. during the period of a collection.

*Results*—The results of sixteen experiments<sup>3</sup> are summarized in Table IV. The average concentration of phosphate in the glomerular urine was 1.4 per cent less than that of plasma. In

<sup>3</sup> The only completed experiments omitted from this table are an early series in which oxalate was used as an anticoagulant, and two in which the glomerular fluid analyses were known to be inexact; in one of this latter pair glomerular fluid was apparently 12 per cent more concentrated, in the other 11 per cent less concentrated than plasma.

TABLE IV

*Inorganic Phosphate in Glomerular Urine and Blood Plasma from Frogs*

Experiment No	Glomerular collection			Plasma P per 100 cc fluid			Glomerular urine P per 100 cc fluid		Difference	Notes
	Pressure	Volume*	Time	Plasma 1†	Plasma 2†	Average	Duplicates	Average		
	mm Hg	cc mm	min	mg	mg	mg	mg	mg	per cent	
1	+15	0 39	37	4 0	4 2	4.1	4 3	4.3	+4 9	No artificial respiration
2	+14	0 13	18	Lost	3 1	3.1	3 2	3.2	+3 2	
3	+5	0 35	57	4 9	5 1	5.0	4 9	4.9	-2 0	
4	+12	0 64	50	3 6	3 9	3.75	3 6, 3 6	3.6	-4 0	
5	+17	0 70	59	4 6	4 4†	4.5	4 3, 4 4	4.35	-3 3	No artificial respiration
6	+10	0 70	45	3 1	3 1	3.1	3 2, 3 3, 3 6	3.35	+8 1	Protein in glomerular urine
7	+5	0 15	43	2 7	2 5	2.6	1 6	1.6	-38 5	No artificial respiration
8	+5	0 33	32	2 7	3 0	2.85	2 6	2.6	-8 8	Protein in glomerular urine
9	+5	0 67	62	3 1	3 2	3.15	3 1, 3 3	3.2	+1 6	" "
10	+12	0 67	50	2 7	3 0	2.85	2 6, 2 8	2.7	-5 3	
11	+10	0 34	62	3 5	3 6	3.55	3 7	3.7	+4 2	" "
										Tubule not blocked
12	+15	1 33	40	3 6	3 4	3.5	3 6, 3 7, 3 8	3.7	+5 7	
13	+11	0 54	63	2 8	2 6	2.7	2 7, 2 9	2.8	+3 7	Same frog as in Experiment 12
14	0	0 18	45	5 2	5 1	5.15	5 1	5.1	-1 0	
15	+12	0 22	29	3 0	3 4	3.2	3 4	3.4	+6 3	Tubule not blocked
16	+12	0 89	28	5 5	5 3	5.4	5 5, 5 6	5.55	+2 8	
Mean	+10	0 51	45			3.65		3.63	-1 4	

\* Approximate.

† Average of duplicate determinations.

‡ Macrodetermination on plasma obtained 30 minutes later, 4.2.

only four experiments did the difference exceed 6 per cent and in only one did it exceed 9 per cent. This approximate equality is

the more striking when it is noted that rates of glomerular collection (and hence presumably of glomerular filtration) and levels of plasma phosphate concentration varied widely in the series. There seems to be no alternative to the conclusion that all of the inorganic phosphate of frog plasma which can be estimated by Kuttner's method is filtrable through the glomerular membrane and can be determined in the glomerular urine.

In ten of these experiments, and in three uncompleted experiments, the bladder became distended with urine during the course of the glomerular collections. Specimens were removed and were shown by analysis to contain phosphate in concentration always higher than that of the plasma; it varied between 4.0 and 14.0 mg. per 100 cc.

#### *Concentration of Phosphates in Glomerular Urine from Necturi*

In 1928, Schmitt and White (10) reported experiments upon *Necturus maculosus* in which the concentration of inorganic phosphates in glomerular urine was found to be only one-fourth to one-third of that in plasma; in bladder urine also it was usually considerably less than in plasma. Since their findings differed so widely from ours in frogs, it seemed important to extend our experiments to include *Necturus*.

The experimental technique was the same as that employed in a study of total molecular concentration of glomerular urine (7). The animals were kept in vigorously oxygenated water for some hours previous to the experiment; the brain was destroyed by crushing the skull with a hemostat; the head and gills were immersed in oxygenated water during the experiment; blood samples were taken from the posterior vena cava 10 minutes before the beginning and 10 minutes after the end of glomerular urine collection.

The results of five satisfactory experiments are shown in Table V (Experiments 1 to 5). Glomerular urine contained inorganic phosphate in concentration from 83 to 101 per cent of that in plasma. In *Necturus*, as in frogs, our analyses disagreed with those of Schmitt and White.

In the hope of settling this disagreement, Dr. White, at the invitation of Professor Richards, came to Philadelphia and collaborated with us in the subsequent experiments. He suggested that

in his earlier experiments the glomerular urine might have been contaminated by phosphate-free saline, dropped on the surface of the kidney, taken into the tubule through the ciliated nephrostome, and sucked back into the capsular space by the negative

TABLE V  
*Inorganic Phosphate in Glomerular Urine and Blood Plasma from Necturi*

Experiment No	Glomerular collection			Plasma P per 100 cc fluid			Glomerular urine P per 100 cc fluid		Difference	Notes
	Pressure	Volume*	Time	Plasma 1†	Plasma 2†	Average	Duplicates	Average		
	mm Hg	c mm	min	mg	mg	mg	mg	mg	per cent	
1	8	0 22	22	5 4	6 5	<b>5.95</b>	6 0	<b>6.0</b>	+0 8	
2	11	0 42	32	3 6	4 3	<b>3.95</b>	3 6, 3 8	<b>3.7</b>	-6 3	
3	5	0 15	63	3 4	4 2	<b>3.80</b>	3 4	<b>3.4</b>	-10 5	Circulation bad Protein in glomerular urine Same <i>Necturus</i> as in Experiment 4
4	5	0 39	34	5 8	6 3	<b>6.05</b>	5 0	<b>5.0</b>	-17 4	
5	5	0 30	22	6 3	6 5	<b>6.40</b>	6 0	<b>6.0</b>	-6 2	
6	8	0 40	22	6 5	5 9	<b>6.20</b>	5 9	<b>5.9</b>	-4 8	
7	11	0 44	8	3 0	3 0	<b>3.00</b>	2 6, 2 8	<b>2.7</b>	-10 0	
8	20	0 33	19	3 9	4 3	<b>4.10</b>	3 8	<b>3.8</b>	-7 3	Protein in glomerular urine
9	15	0 24	11	3 2	3 6	<b>3.40</b>	3 2	<b>3.2</b>	-5 9	
10	23	0 08	6	2 9	3 4	<b>3.15</b>	3 2	<b>3.2</b>	+1 6	
Mean.	11	0 28	24			<b>4.6</b>		<b>4.3</b>	-6 6	

In Experiments 6 to 10 the glomerular urine collections were made by Dr. H. L. White. All of the analyses were made by the authors.

\* Approximate.

† Average of duplicate determinations.

pressure in the collecting system. This possibility was seen to be realized in tests in which trypan blue was applied to the surface of the kidney. Hence, in the experiments which Dr. White made, no saline was dropped on the kidney surface and positive pressure was maintained in the glomerular urine-collecting system. His

technique differed essentially from ours only in that he anesthetized the animals with urethane. The results of five experiments made by Dr. White (Table V, Experiments 6 to 10) agreed with ours; the phosphate concentration of glomerular urine was 90 to 102 per cent of that of plasma. In Experiments 9 and 10, after the first collection was completed, the pipette was reinserted into the same capsule and a second collection made by a technique similar to that used in White's earlier experiments: phosphate-free saline was dropped on the kidney and glomerular urine was collected with negative pressure. Whereas in both experiments the first glomerular urine collected contained 3.2 mg. of phosphate per 100 cc., the second collection in Experiment 9 contained 1.5; in Experiment 10, 1.3. Dr. White was convinced that the low figures for glomerular urine phosphate in his previous experiments were the results of technical error, and has published the above results with a revision of his previous conclusions (19, 20).

In these ten experiments the mean value of phosphates in glomerular urine was 4.3; in plasma, 4.6 mg. per 100 cc. The agreement is not as close as in the experiments with frogs. The tendency of plasma phosphate in *Necturus* to rise during the course of an experiment makes the comparison with glomerular urine less reliable; and the sluggish glomerular circulation often encountered in *Necturus* makes it difficult to be certain that the collected fluid is newly formed and not contaminated with fluid from the tubule. For these reasons we attach little significance to this difference and conclude that in *Necturus*, as in frogs, the concentration of inorganic phosphate in glomerular urine is that of a filtrate from plasma.

In five of the six specimens of bladder urine obtained from pithed *Necturi*, phosphate concentration was higher than that of plasma (average, 9.1 mg. per 100 cc.). This was also true of frogs. In White's earlier experiments the concentration of phosphates in bladder urine was usually less than that in plasma. We have no explanation to offer for this discrepancy.

*Increase in Plasma Phosphate Produced by Carbon Dioxide and Exercise*—Pithed *Necturi* showed an increase in plasma phosphate concentration of as much as 1.0 mg. per 100 cc. per hour over a period of 2 hours. Either asphyxia (accentuated by circulatory failure) or exertion might have been responsible for



the observed change (21-23). The possibility was investigated in frogs.

Two frogs, placed for 70 minutes in an atmosphere of 10 per cent carbon dioxide, showed increases in plasma phosphate concentration of from 2.1 to 2.6 and from 2.1 to 3.7 mg. per 100 cc. Five frogs were exercised to complete exhaustion for 20 minutes; specimens of cardiac blood or lymph<sup>4</sup> were obtained at the beginning and end of the experiments and microphosphate analysis made upon them. The rise in phosphate concentration

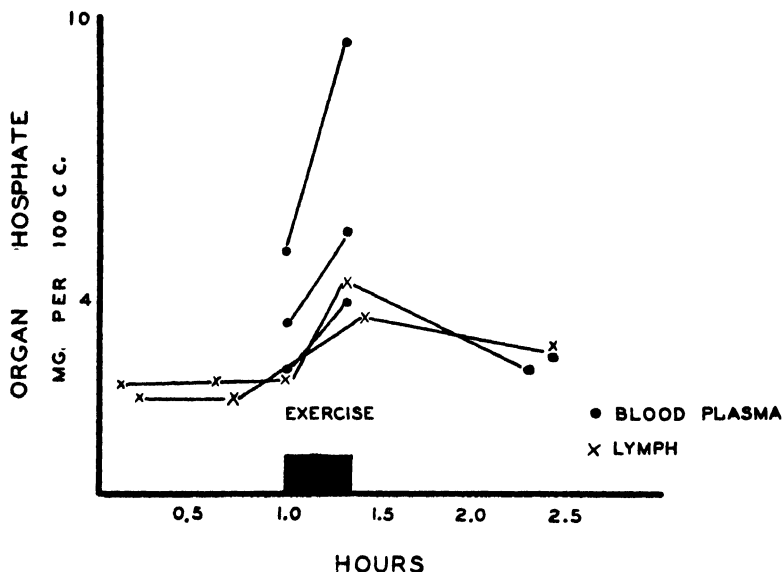


FIG. 1. Effect of exercise on inorganic phosphate content of blood plasma and lymph of frogs.

was pronounced in every case, and varied from 50 to 84 per cent of the control value (Fig. 1). We concluded that both phenomena are capable of affecting the phosphate concentration of frog plasma, and that either or both might have been concerned in the changes which we had observed in *Necturi*.

<sup>4</sup> The use of lymph was made necessary by the difficulty of obtaining consecutive specimens of cardiac blood without unduly disturbing the frog, and appeared justified by our demonstration of the equality of phosphate concentrations of this fluid and plasma (18).

*Total Molecular Concentration of Glomerular Urine of Frogs and Necturi*—Barger's capillary method (7) for the determination of total molecular concentration was employed on glomerular urine from one frog and five *Necturi*. The comparison was against plasma. Collections from the *Necturi* were made by Dr. H. L. White in this laboratory and the determinations by Dr. Elizabeth T. Krick. In each instance the concentration of glomerular urine and plasma was found to be the same within the limits of error of the method (approximately 5 per cent). These experiments are not reported in detail since their results simply confirm those of similar experiments previously performed in this laboratory.

#### SUMMARY

Kuttner's modification of the Bell-Doisy method for the determination of inorganic phosphates has been adapted to amounts of fluid as small as 0.08 c.mm. containing less than 0.000001 mg. of phosphate P. The mean of the errors of a series of determinations upon aqueous phosphate solutions of known concentration was +0.1 per cent; the mean deviation, 2.5 per cent. Applied to plasma, the method gave results closely similar to those obtained by the macromethod. A small fraction of phosphate added to phosphate-free plasma appeared to escape determination. In frog plasma, *Necturus* serum, horse serum, and human serum, all of the inorganic phosphate which can be determined by Kuttner's method is filtrable through cellophane. Lymph collected from the frog's web contained about 94 per cent as much phosphate as did plasma.

Application of the microadaptation of Kuttner's method to glomerular urine and plasma from frogs showed the phosphate concentration of the two fluids to be approximately the same. Glomerular urine from *Necturi* yielded slightly lower values than plasma. Reasons are advanced for believing that this difference is not significant.

Bladder urine from frogs and *Necturi* contains inorganic phosphate in concentration from 1 to 4 times that of plasma. Therefore, it is our present belief that glomerular filtration is adequate to account for all of the inorganic phosphate normally excreted in the urine of both species.

Grateful acknowledgment is made to Dr. H. L. White for his collaboration in experiments which settled the conflict between his results and those described in this paper.

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## QUANTITATIVE STUDIES OF THE COMPOSITION OF GLOMERULAR URINE

### XI. THE CONCENTRATION OF CREATININE IN GLOMERULAR URINE FROM FROGS DETERMINED BY AN ULTRAMICRO- ADAPTATION OF THE FOLIN METHOD\*

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In Rehberg's development of Cushny's theory of urine formation (1), it is assumed that no reabsorption of creatinine from the glomerular filtrate occurs during its passage through the tubule. On the basis of this assumption, the concentration ratio of creatinine has been used in calculating the volume of the glomerular filtrate and the amounts of other dissolved substances and of water which are reabsorbed. Detailed, accurate information concerning the passage of creatinine through the kidney is therefore particularly important.

Success in adapting colorimetric methods for determining uric acid (2), glucose (3), and phosphates (4) to minute amounts of fluid encouraged the belief that a similar refinement of method for the estimation of creatinine could be accomplished. The data contained in this paper show that as little as 0.00001 mg. of creatinine dissolved in 0.5 c.mm. of fluid can be determined with reliable accuracy by a capillary tube adaptation of the Folin method (5), and that the concentration of creatinine in glomerular urine of frogs is the same as that in the blood plasma from which it is derived.

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† National Research Council Fellow in Medicine, 1930-32.

*Analytical Method*

*Development*—The work has proceeded in two separate stages. In the first, the Folin method as described by Folin and Wu (5) was adopted without changes other than those incident to the use of minute volumes in capillary tubes. These included not only the application of the manipulative technique described by Richards, Bordley, and Walker (6), but two other procedures designed to overcome difficulties which had not been encountered in the ultramicromethods for estimating uric acid, glucose, or phosphates. The first of these concerned the color comparisons in capillary tubes. When creatinine solutions of the standard range are mixed in capillary tubes with Folin's reagent in the proportion of 2:1, the colors resulting from the creatinine compound are so masked by that of the sodium picrate that accurate comparisons cannot be made by daylight or by ordinary electric light. The interposition of a screen of saturated picric acid solution, or, more conveniently, of a sheet of straw-colored gelatin, between the source of light and the tubes so obliterates the yellow and brings out the red that accurate comparisons can be made consistently.

The second difficulty lies in the fact that when a minute amount of Folin's reagent (5 volumes of saturated picric acid plus 1 volume of 10 per cent NaOH) is allowed to stand in a capillary tube, spontaneous deepening of color occurs at a faster rate and to a far greater extent than is the case when a large volume of the reagent stands in a test-tube. This change is greatly intensified and accelerated by daylight; it is not altered by using quartz capillary tubes instead of glass. In order to overcome this difficulty all of the manipulative procedures were conducted in a darkened room, illuminated only by one electric light placed in front of the microscope and equipped with a screen of *amber* gelatin. The time consumed in preparing the tubes and in making the color comparisons was made as short as possible and the various steps were conducted according to a prearranged time schedule.

Even with these precautions a systematic positive error was present, apparently due to progressive darkening of the reagent in the capillary tube, not wholly abolished by exclusion of light. The error was so consistent both in sign and magnitude that it was decided to accept it and proceed with analyses of glomerular urine.

The error was not present in analyses of tungstic acid filtrates from blood plasma made in identical fashion. Ten satisfactory experiments were made in which glomerular urine and plasma were collected from frogs and analyzed for creatinine. The average difference between the values of creatinine in the glomerular urine and the plasma was almost identical with the average error encountered in the analysis of solutions of pure creatinine. This indicated identity of concentration of creatinine in the two fluids.

At the outset of the second stage of the work, another series of known creatinine solutions was analyzed by the same method with essentially identical results. Additional evidence was obtained that darkening of Folin's reagent in a capillary tube proceeds in a darkened room at a rate which appeared to be sufficient to account for the error. The degree of alkalinity of the reagent was found to be an important factor. Consequently, it was decided to abandon the use of the mixed reagent and to introduce the constituents of the reagent into the capillary tube as separate columns, along with the creatinine solution to be analyzed. Sixteen analyses of known solutions, each in duplicate, gave results the average of which coincided with the true value. The systematic error had been eliminated. Five experiments with frogs were then made. The differences between creatinine found in glomerular urine and in plasma were those expected from ordinary analytical errors.

In the following pages the method first used will be referred to as Method A. It is unnecessary to describe it further than to say that a column of the creatinine solution to be analyzed, 5 mm. long, was drawn into a capillary tube; then a column of Folin's reagent 2.5 mm. long. Mixture was accomplished by centrifugation, Color comparison was made with standard mixtures prepared in test-tubes. All operations were conducted in a darkened room as rapidly as possible.

The second method, Method B, which eliminates the error of the first is as follows:

*Description of Method. Solutions*—Standard creatinine solutions were made by dissolving Pfanstiehl's creatinine in 0.01 N HCl with toluene as preservative. The concentrations used were 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 6.0 mg. per 100 cc.

*Picric acid*, C.P., was purified according to Benedict's second

method (7). Tested by the Folin and Doisy method (8) it gave a reading of 12.6 and hence was acceptable according to their standards.

*Sodium Hydroxide, 10 Per Cent*—Folin's reagent was prepared freshly before use, by mixing 5 volumes of saturated picric acid solution with 1 volume of 10 per cent NaOH (Merck's reagent, "from sodium").

*Procedure*—A capillary tube, 0.35 mm. inside diameter, was connected with the water manipulator and mounted on the microscope stage in the manner described in Paper VII of this series (6). A column of each of the three solutions to be mixed was drawn into the end under the microscope and kept separate from the others by columns of air: first, saturated picric acid, 25 scale divisions of the ocular micrometer; second, creatinine solution to be analyzed, 60 scale divisions; last, 10 per cent NaOH, 5 scale divisions. 60 scale divisions were approximately equivalent to 0.5 c.mm. As soon as the fluids had been drawn in so that the last was about 1 cm. from the end of the tube, the distal part of the tube containing them was broken off, its ends sealed in the flame, and laid aside in a closed box until other tubes of the series had been similarly prepared. This having been done, the following operations were performed in the darkened room.

The standard creatinine color mixtures were made in eight test-tubes. To 2 cc. of a standard solution previously measured into each, 1 cc. of Folin's creatinine reagent was added. These mixtures were prepared as rapidly as was compatible with accuracy, and immediately thereafter the capillary tubes, each containing a solution to be analyzed and the separate components of the creatinine reagent, were placed together in the centrifuge and the fluid columns mixed by repeated centrifugation. The color comparisons were begun 10 minutes after the first centrifugation.

During this 10 minutes the contents of each capillary and a portion of each standard mixture were transferred to pieces of capillary tubing known by measurement to be of the same diameter (0.35 mm.) and the ends sealed with plasticine. Each was placed in a labeled space on a milk glass plate where the color comparisons were to be made.

Color comparisons were made in a dark room under a 200 watt

bulb equipped with a filter of straw-colored, sheet gelatin.<sup>1</sup> The color produced by 2.0 mg. of creatinine per 100 cc. is about the palest which can be read reliably in capillary tubes of the dimensions used. The 6.0 mg. standard gives a relatively deep orange-red. The most advantageous colors are those produced in standards from 2.5 to 5.0 mg. per 100 cc. When the color of an unknown failed to match any standard exactly, the difference was estimated to 0.1 mg. per cent.

The color readings are difficult and require practise. However, two experienced persons, independently comparing the colors in twenty-nine consecutive estimations, made the same reading in nine, differed by 0.1 mg. per cent in fourteen, by 0.2 mg. per cent in four, and by 0.3 mg. per cent in two. These differences amounted to 2 per cent or less in twenty-three instances; 4 to 7 per cent in six. Independent readings have never differed by more than 7 per cent.

*Preliminary Determinations and Dilutions*—When the concentration of a solution to be analyzed was unknown, it was compared in a preliminary determination with 2.0 and 5.0 mg. per cent standards. Solutions which contained more than 5.0 mg. per cent were routinely diluted 2.5 times. We have not analyzed solutions so concentrated that a 2.5-fold dilution would not bring them into the range of the standards. The dilutions were made by introducing into the mixing capillary a measured column of the unknown and increasing its length to 2.5-fold by the addition of 0.01 N HCl.

In these dilutions the attempt was made in each instance to attain the 0.01 N acidity of the standard solutions. Unknown solutions of pure creatinine were made up in 0.01 N acid and were therefore diluted with 0.01 N acid. Ultrafiltrates and glomerular fluids were diluted 2.5 times with 0.02 N HCl; here the proper acidity could only be approximated because of ignorance of the degree of alkalinity of the original material. The acidity of the tungstic acid filtrates from plasma was that which resulted from 2.5-fold dilution with tungstic acid.

*Analysis of Frog Plasma*—In the control tests of method, creat-

<sup>1</sup> One of us prefers to place a smaller electric light bulb and the color screen *under* the milk glass plate. Sheets of straw-colored gelatin are obtainable from dealers in theatrical lighting supplies.



inine determinations by Method A were made both on ultrafiltrates and on tungstic acid filtrates of plasma. Only tungstic acid filtrates were used in comparing the creatinine concentration of glomerular urine and plasma.

Analysis of normal frog plasma gave a result equal to that of a solution containing about 0.6 mg. of creatinine per 100 cc. It was therefore necessary to work with plasma to which creatinine had been added or with plasma taken from frogs previously injected with creatinine. In a study of the method, the procedure most frequently followed was to dialyze pooled plasma from a number of frogs against 0.7 per cent NaCl solution until it gave no color reaction with alkaline picrate (about 72 hours). Creatinine was then added in known amount to the dialyzed plasma. Tungstic acid filtrates were prepared by precipitating the plasma proteins in capillary tubes with the tungstic acid mixture (6). Because of this 2.5-fold dilution we have been unable to estimate the creatinine in plasma containing less than 5 mg. per cent of creatinine.

The ultrafiltrates which were analyzed were prepared by filtration through collodion membranes at a pressure of 90 mm. of Hg or through cellophane at 200 to 250 mm. Tests for protein in the ultrafiltrates were negative.

*Results of Analyses of Known Solutions*—In every instance the analyst was ignorant of the concentration of the solution until the analysis was completed. Two groups of analyses were made by Method A. In one, ten solutions, the concentrations of which ranged from 3.7 to 9.7 mg. per 100 cc., were analyzed, nine in duplicate. The greatest error was +8 per cent; the least, +1 per cent; average, +4.8 per cent. In the second, fifteen solutions, ranging in concentration from 2.5 to 5.3 mg. per cent, were analyzed in duplicate. The greatest error was +11.3 per cent; the least, 0; average error, +4.8 per cent. In only two instances was the difference between duplicates greater than 0.2 mg. per 100 cc.; the average difference was 0.1 mg. These results are shown in Table I.

The second series of analyses of known solutions was made by Method B. Sixteen solutions, the concentrations of which ranged from 2.1 to 5.35 mg. per cent were analyzed in duplicate. The greatest error was +10.1 per cent; the mean of the errors, +0.02 per cent; average deviation from the mean, 2.75 per cent. These results are also shown in Table I.

TABLE I

*Determination of Creatinine in Pure Solutions*

Average volume of solution analyzed = 0.5 c.mm.

Method A				Method B			
Experiment No	Concentration of solution		Error	Experiment No	Concentration of solution		Error
	Known	Found*			Known	Found*	
	mg. per 100 cc.	mg. per 100 cc.			mg per 100 cc.	mg per 100 cc.	
Group I				1	2 6	2 5	-3 8
1	4 3	4 5	+5	2	3 10	3 15	+1 6
2	3 7	3 8	+3	3	4 20	4 35	+3 6
3	8 8	9 5	+8	4	2 20	2 25	+2 3
4	9 4	9 9	+5	5	5 35	5 20	-2 8
5	9 7	10 2	+5	6	2 4	2 3	-4 2
6	7 8	8 3	+6	7	5 00	4 85	-3 0
7	9 2	9 9	+8	8	3 0	2 9	-3 3
8	8 3	8 4	+1	9	4 95	5 45	+10 1
9	9 2	9 6	+4	10	2 30	2 25	-2 2
10	8 6	8 9	+3	11	5 25	5 20	-1 0
Mean.			+4 8	12	3 25	3 20	-1 5
Average deviation from mean..			1 6	13	4 4	4 6	+4 5
Group II				14	2 1	2 1	0
1	3 45	3 45	0	15	3 5	3 5	0
2	3 55	3 50	-1 4	16	4 5	4 5	0
3	3 30	3 55	+7 6	Mean ..			+0 02
4	3 40	3 75	+10 3	Average deviation from mean			2 75
5	4 50	4 75	+5 6				
6	2 50	2 55	+2 0				
7	4 5	4 6	+2 2				
8	3 10	3 45	+11 3				
9	5 10	5 25	+2 9				
10	5 3	5 0	-5 7				
11	2 8	3 0	+7 1				
12	3 50	3 75	+7 1				
13	4 5	4 8	+6 7				
14	4 9	5 3	+8 2				
15	2 6	2 8	+7 7				
Mean .			+4 8				
Average deviation from mean..			3 9				

\* Average of duplicates.

*Results of Analyses of Plasma Filtrates*—Dialyzed frog plasma was prepared, divided into six samples, and creatinine added to make concentrations varying from 6.9 to 11.2 mg. per cent. Tungstic acid filtrates were prepared from these in capillary tubes as has been described. The analyses were conducted by Method A. Of the six analyses, each made in duplicate, the greatest error was  $-2.1$  per cent. The mean of the errors was  $-0.16$  per cent; the average deviation from this,  $\pm 1.7$  per cent (Table II). The absence from these determinations of the systematic error present

TABLE II

*Frog Plasma: Creatinine Added in Known Amounts. Analyses of Tungstic Acid Filtrates and Ultrafiltrates by Capillary Tube Method A*

Experiment No	Creatinine			Difference (3-2)	Difference (4-2)
	Added	Found*			
		In tungstic acid filtrate (3)	In ultra- filtrate (4)		
(1)	(2)	(3)	(4)	(5)	(6)
	<i>mg per 100 cc</i>	<i>mg per 100 cc</i>	<i>mg per 100 cc</i>	<i>per cent</i>	<i>per cent</i>
1	6 9	6 8	7 2	-1 4	+4 3
2	9 6	9 4	9 6	-2 1	0
3	11 2	11 4	12 1	+1 8	+8 0
4	7 8	7 8	8 1	0	+3 8
5	9 1	9 0	9 3	-1 1	+2 2
6	10 8	11 0	11 6	+1 8	+7 4
Mean				-0 16	+4 3

\* Average of duplicates.

in the analyses of pure creatinine solutions by Method A was apparently due to inhibition of the spontaneous darkening of the reagent brought about by the trace of tungstic-sulfuric acid mixture which was taken up by the reagent as it was drawn into the capillary tube previously wetted by the plasma filtrate.

An ultrafiltrate was made from each of the six plasma samples used in the above analyses and the creatinine estimated in each in duplicate by the above method. The greatest error was  $+8.0$  per cent; the mean of the errors was  $+4.3$  per cent; the average deviation from this,  $\pm 2.3$  per cent.

This difference in the behavior of tungstic acid filtrates from

that of ultrafiltrates in the microdetermination of creatinine does not appear in macroanalyses by the Folin method. This was shown in six experiments, the results of which are given in mg. per cent, as follows:

Experiment No	1	2	3	4	5	6
Creatinine in tungstic acid filtrate	6 4	7 0	7 4	3 1	3 5	6 5
“ “ ultrafiltrate	6 7	7 0	7 4	3 0	3 5	6 3

The conclusions to be drawn from these series of analyses are obvious. When Folin's alkaline picrate reagent is used in the capillary tube technique, analysis of solutions of pure creatinine and of ultrafiltrates from plasma containing added creatinine gives results which show a consistent positive error amounting to about 5 per cent. It must be assumed that this error will appear in analyses of glomerular urine made by the same method. The error is absent from similar determinations of creatinine in tungstic acid filtrates; it can be eliminated from determinations of creatinine in pure solutions by introducing the constituents of Folin's reagent separately into the capillary tube.<sup>2</sup>

#### *Analysis of Glomerular Urine and Plasma from Living Frogs*

Two groups of experiments have been made: the first were made by Method A between April 13 and June 16, 1932, on twelve female frogs (one *Rana catesbeiana*, eleven *Rana pipiens*); two are not reported: one because the creatinine values were beyond the range of our standards, the other because of possible contamination of glomerular fluid with phenol red (see below). The analyses were made by Method A. Hence, the figures for creatinine in glomerular urine must be regarded as about 5 per cent too high; those for creatinine in plasma (tungstic acid filtrates) as correct.

The second group of five experiments (*Rana pipiens*) was made in February, 1933. The analyses were made by Method B.

The results of all the analyses are shown in Table III.

<sup>2</sup> The obvious indication supplied by analyses of tungstic acid plasma filtrates that the addition of a minute amount of tungstic acid to Folin's reagent retards the spontaneous darkening of color in capillary tubes was borne out by direct tests; it seemed scarcely worth while to attempt to utilize this fact in the elimination of the error.

## 264 Glomerular Urine Composition. XI

The following details of experimental technique should be specified.

30 minutes before the brain was destroyed by hemostat, a 0.2 per cent solution of creatinine in Ringer's solution was injected sub-

TABLE III

*Creatinine in Glomerular Urine and Blood Plasma of Frogs*

Method A was used in the analyses of Experiments 1 to 10; Method B in Experiments 11 to 15

Experiment No	Glomerular urine		Time of collection		Concentration per 100 cc of creatinine in				Difference
	Time of collection	Collection pressure	Blood 1	Blood 2	Plasma 1†	Plasma 2†	Average	Glomerular urine	
	min	mm Hg	min *	min *	mg	mg	mg	mg	per cent
1	60	+10	10	50	12 3	12 4	12.35	13.35†	+8
2	45	+5	10	50	5 3	5 15	5.23	5.4†	+3
3	60	+8	10	50	6 3	5 6	5.95	6.2	+4
4	47	+16	12	45	7 9	7 85	7.88	8.1	+3
5	48	+8	10	50	12 5	11 6	12.05	12.9	+7
6	44	+7	12	32	10 7	10 0	10.35	11.2†	+8
7	109	+14	23	87	6 9	6 5	6.7	6.6	-2
8	105	+9	23	85	6 3	5 65	5.98	6.3	+5
9	113	+1	16	95	11 0	9 6	10.3	10.5	+2
10	124	+3	17	107	10 3	7 9	9.1	9.45†	+4
Mean									+4 2
11	120	-10	11	115	10 5	10 5	10.5	10.6	+1 0
12	60	+8	11	53	12 5	11 9	12.2	11.6	-4 9
13	60	+9	21	48	10 0	9 4	9.7	10.0†	+3 1
14	15	+3	10		12 0		12.0	12.4	+3 3
15	91	+2	10	98	11 7	10 1	10.9	10.8	-0 9
Mean									+0 3

\* Minutes after beginning of collection of glomerular urine.

† Analyses were made in duplicate.

cutaneously, in amount to give 0.05 to 0.12 mg. of creatinine per gm. of body weight.

The usual identification of the tubule by intracapsular injection of phenol red in order to make possible its obstruction was omitted. The presence of the merest trace of this dye in the fluid

influences the creatinine reading.<sup>3</sup> In nine experiments, therefore, the obstructing rod was pressed on the surface of the kidney at a point corresponding to that at which the neck of the tubule is usually seen when dye is injected. In six of these the correctness of the block was verified by intracapsular injection of phenol red at the end of the experiment; no attempt at verification was made in two; it was unsuccessful in one. In the six experiments in which no block was attempted the mercury bulb of the collecting pipette system was raised to over 200 mm. above the kidney before the collection began, and lowered very cautiously and slowly during the collection to a minimum level of from 1 to 14 mm. above the surface of the kidney. The clear space between capsule and tuft was never allowed to diminish perceptibly. The relatively low pressures against which glomerular urine was collected in Experiments 9, 10, 11, 14, and 15 were justified by the unusual narrowness of the tips of the collecting pipettes (5 to 7 $\mu$ ). Under these conditions, rates of collection were unusually slow and in six experiments not enough fluid for duplicate determinations was obtained. Volumes collected ranged from 0.15 to 0.5 c.mm.

The glomerular fluid was transferred to the mixing capillary under oil and diluted 2.5 times with 0.02 N HCl.

Arterial blood was taken from the ventricle into a capillary pipette shortly after the beginning and shortly before the end of a glomerular urine collection. A minute amount of dry sodium oxalate in the pipette prevented clotting. Immediately after the blood collection, the pipette was sealed, and the plasma separated in the centrifuge. The tungstic acid "filtrates" were prepared in capillary tubes. The dilution in the precipitation was 2.5 times.

All analyses of plasma and five of glomerular urine were made in duplicate. The duplicates are not recorded in Table III since they always agreed within 6 per cent and usually within 3 per cent. In each experiment the four plasma tubes and the glomerular urine tube (or tubes) were compared with the same set of standards. The duplicate tubes of the first plasma were prepared before, and those of the second plasma after, the preparation of the glomerular urine tubes. In Experiments 2 to 5 the standards were prepared in capillary tubes; in all the others, in test-tubes.

<sup>3</sup> When a creatinine solution was made to contain phenol red in concentration one-thousandth of that used in identification of the tubule, there was an apparent increase in the creatinine content of about 1.0 mg. per cent.

In eight experiments, the glomerular urine was tested for protein by the capillary tube method (6). In Experiments 1, 5, 7, and 8 it was diluted 2.5 times; in Experiments 2, 6, 11, and 13 it was undiluted. In Experiments 6 and 11 the protein concentration was like that of a 1:200 dilution of frog plasma; in Experiment 13 it was equivalent to 1:500 plasma; all the other tests were negative.

### *Results*

In Experiments 1 to 10 in which, because of systematic analytical error, it was expected that the determinations of creatinine in glomerular urine would yield results about 5 per cent too high, the average of the observed differences between glomerular urine and plasma was +4.2 per cent; in Experiments 11 to 15 in which the systematic error was avoided, the average of the observed differences was +0.3 per cent. The variations from these averages were relatively small. Hence we conclude that creatinine, like uric acid, reducing substance, phosphates, urea, and such dyes as have been studied, passes through the glomerular membrane of the frog at the same rate as the protein-free fluid in which it is dissolved.

*Creatinine Concentration Ratio*—In a number of experiments creatinine was determined in bladder or ureteral urine and the concentration ratio calculated. In one no creatinine had been injected: preformed creatinine in the plasma was 0.6 mg. per cent; in urine, 4.3 mg. per cent; concentration ratio, 7.2. In the others creatinine was injected in varying amounts. The concentration ratios found were 2.3, 2.5, 3.2, 5.5, 12.4. They are very similar to the concentration ratios of uric acid in frogs (2). The figures are of interest in connection with the results of Höber's recent experiments with perfused kidneys (9). When creatinine was added to the aortic perfusion fluid, its concentration in the urine was about double that in the perfusion fluid; when it was added to the renal portal perfusion fluid its concentration in the urine was less than that in the perfusion fluid. In this respect its behavior differed from that of uric acid. While our experiments were not designed to test this, they fail to give any indication of a difference between creatinine and uric acid in respect of its passage through the kidney of the living frog.

## SUMMARY

1. The reactions and color comparisons of the Folin method for the estimation of creatinine can be conducted in capillary tubes. Thus the method is made applicable to volumes of fluid of approximately 0.5 c.mm. containing from 0.00001 to 0.00003 mg. of creatinine. When Folin's alkaline picrate reagent is used the adapted method contains a systematic error apparently due to spontaneous change in the color of the creatinine reagent upon standing in a capillary tube. When a standard schedule of manipulations is rigidly followed, this error is consistent in sign and magnitude and on the average amounts to less than 5 per cent. The error was eliminated by keeping the constituents of Folin's reagent separate until the instant of mixture with the creatinine solution.

2. Analyses of glomerular urine and plasma from frogs which had been injected with pure creatinine showed that the concentration in the two fluids is the same.

3. Bladder and ureteral urine of these frogs contained creatinine in concentrations from 2.3 to 12.4 times those of the plasma.

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**COMPARISON OF THE CHEMICAL COMPOSITION OF  
AQUEOUS HUMOR, CEREBROSPINAL FLUID, LYMPH,  
AND BLOOD FROM FROGS, HIGHER ANIMALS,  
AND MAN**

**REDUCING SUBSTANCES, INORGANIC PHOSPHATE,  
URIC ACID, UREA\***

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In the course of the quantitative studies of glomerular urine which have long been in progress in this laboratory, analytical methods requiring exceedingly minute amounts of fluid have been developed for a number of constituents of blood and urine. It seemed important to extend the application of these methods to certain other body fluids for the sake not only of the information which might be gained, but also of the influence which the results might have upon the credibility of the analyses of fluid from single renal units.

The fluids chosen for study are lymph, cerebrospinal fluid, and aqueous humor. The analyses to which they have been subjected include determination of reducing substances, inorganic phosphate, uric acid, and urea. The results of analyses of lymph and cerebrospinal fluid of frogs were quite in accord with those of the majority of investigators who have analyzed these fluids in mammals. In the case of the aqueous humor of frogs, however, our results were so at variance with accepted ideas of the com-

\* The expenses of this investigation were defrayed in large part from a grant by the Commonwealth Fund. A preliminary report of these experiments was made before the American Society of Biological Chemists at Philadelphia, April 29, 1932 (*J. Biol. Chem.*, **97**, lxxii (1932)).

position of this fluid that the observations were extended to the aqueous humor of higher animals and man with results which confirmed the findings upon frogs. Despite the fact that the study has been restricted to the four substances mentioned above, the results make it appear extremely unlikely that either cerebrospinal fluid or aqueous humor is formed by purely physical processes of filtration or dialysis.

### *Analytical Methods*

The ultramicrocolorimetric technique described by Richards, Bordley, and Walker (1) was employed in the determinations of reducing substances (2), inorganic phosphate (3), and uric acid (4). Approximately 0.2 c.mm. of fluid was used in each determination and all determinations were made in duplicate. The low protein content of cerebrospinal fluid and aqueous humor of frogs, higher animals, and man made routine deproteinization for the phosphate analysis unnecessary. Frog lymph was analyzed in the same way as frog plasma. In the phosphate analyses of mammalian blood plasma, the quantity of trichloroacetic acid used in deproteinization was double that used in frog plasma; in the sugar determinations mammalian plasma was subjected to preliminary dilution with 1 volume of 0.9 per cent sodium chloride. The accuracy of these micromethods, as described in the original papers, approaches closely that of the macromethods in common use. In seven of the experiments upon the reducing substances in mammalian aqueous humor, in addition to the micromethod (2), analyses by the Hagedorn-Jensen method (5) were made by Dr. B. B. Westfall of this laboratory. All but two of the urea determinations were made by the aeration-titration method of Van Slyke and Cullen (6), with 1.0 cc. of aqueous humor and plasma in the experiments upon dogs, 0.5 cc. in those on rabbits; in five experiments the analyses were made by Dr. K. A. Elsom of this laboratory; in the two instances specified in Tables IV and V the amount of dioxanthidrolurea precipitated by centrifugalization of 0.6 c.mm. of fluid and 0.7 c.mm. of glacial acetic acid and xanthidrol in a small glass capillary tube was taken as a measure of the amount of urea present; to these two analyses less weight need be given than to the other urea analyses.

## EXPERIMENTAL

*Frogs*

*Lymph*—0.01 to 0.02 cc. of lymph was collected from the web of a frog's foot by inserting the finely drawn tip of a glass capillary pipette into the lymph vessel at the margin of one of the toes. The fluid was clear, colorless, and devoid of red blood corpuscles. It was obtained within 1 to 4 minutes after blood had been collected by cardiac puncture, and contained about half as much protein as the plasma.

*Cerebrospinal Fluid*—The experiments were performed between November, 1931, and April, 1932, upon healthy *Rana pipiens*. Cerebrospinal fluid was collected from the cisterna magna by pushing the finely drawn tip of a Pyrex glass capillary pipette (0.6 mm. internal diameter) through the atlanto-occipital ligaments. An oval section of skin and the vertebral muscles immediately above the site of puncture were removed by cautery before the collection was made. When the pipette point entered the cistern about 0.02 cc. of clear, colorless fluid rose in the pipette. This amount would have been sufficient for more than a dozen analyses with the technique employed. The collection was finished within from 30 seconds to 2 minutes after the frog was removed from the cage; within 1 or at most 2 minutes thereafter the skull was crushed by hemostatic forceps, the chest wall opened, and a specimen of blood obtained by cardiac puncture.<sup>1</sup> It is believed that these time relations, and those observed in the collection of aqueous humor, were such as to obtain normal plasma sugar values and to avoid the fictitious differences between these fluids and plasma which might have been caused by delaying the blood collection.

*Aqueous Humor*—The brain was crushed and blood collected by cardiac puncture within 35 to 90 seconds after the animal was removed from the cage.<sup>2</sup> When venous blood was used it was

<sup>1</sup> In Experiments 15, 20, 23, and 24 of Table I, the interval between collection of cerebrospinal fluid and plasma slightly exceeded 2 minutes. A 45 minute interval in Experiments 12, 17, 18, and 20 of Table II was considered relatively unimportant because of the stability of phosphate concentration in the blood of pithed frogs (3).

<sup>2</sup> In experiments where cerebrospinal fluid also was collected, the cardiac puncture was delayed about 1 minute.

obtained from the posterior vena cava. Within 30 seconds thereafter the aqueous humor was collected in a pipette similar to that employed for lymph and cerebrospinal fluid. Its tip was thrust through the cornea into the anterior chamber of the eye, the angle and depth of the thrust being so directed as to avoid the lens. As soon as the point penetrated the cornea about 0.01 cc. of clear, colorless fluid entered the pipette, which was then withdrawn; about 0.02 cc. was left in the anterior chamber.

### *Healthy Higher Animals*

*Aqueous Humor*—With exception of the rabbits of Experiments 7, 9, and 10 (Table IV), the animals had not been fed for at least 12 hours before the experiment. In six instances they were anesthetized with barbital before collections of blood and aqueous humor were made; in twenty-five instances venipuncture was performed without anesthesia, as speedily and with as little disturbance of the animal as possible. Oxalated plasma or serum was used for the analyses. Aqueous humor was collected immediately following venipuncture and after cocainization of the cornea in the unanesthetized animals. When macroanalyses were to be made, the cornea was punctured by the sharp point of a glass pipette and the anterior chamber rapidly evacuated; about 0.6 cc. could be obtained from each eye of a dog, about 0.25 cc. from each eye of a rabbit. When only microanalyses were to be made, 0.02 cc. was collected in the manner described for frogs.

### *Man*

*Aqueous Humor*—The practicability of these experiments was suggested by Dr. James Bordley, 3rd. Four specimens of aqueous humor were obtained for us by Dr. Francis H. Adler in the course of preliminary iridectomies for cataract at the Wills Eye Hospital. The patients had not been fed for at least 6 hours previous to the operation and no general anesthetic was used. The pathological condition of the lens was believed to be the only abnormality. The volume of each specimen was approximately 0.02 cc. In addition to the analyses for reducing substances and phosphates which were made upon all, two of the specimens were analyzed for uric acid by Dr. Bordley, and one of these for urea by the xanthidrol method. The comparisons were made against serum

obtained by venipuncture within 5 minutes of the time of aqueous humor collection, and against protein-free ultrafiltrates subsequently prepared from these sera by means of cellophane membranes. Analyses were begun within 1 hour after the collections were finished.

### *Depancreatized Dogs*

*Cerebrospinal Fluid and Aqueous Humor*—Dr. I. S. Ravdin of the Department of Surgical Research removed the pancreas from each of three dogs under amytal anesthesia. No insulin was given and the animals died within 90 hours. 48 and 72 hours after operation, specimens of venous blood, cerebrospinal fluid, and aqueous humor were collected for ultramicroanalysis. The first specimen of aqueous humor was collected from the left, the second from the right eye. Blood and aqueous humor were taken before the brief period of ether anesthesia which was usually required for the collection of cerebrospinal fluid.

### RESULTS

*Lymph*—We have compared the concentrations of reducing substances (eight experiments) and inorganic phosphate (six experiments) in web lymph and arterial blood plasma from frogs. These comparisons, summarized in Tables I and II, show that lymph contains 97 per cent of the plasma reducing substances, 94 per cent of the plasma phosphate.

*Cerebrospinal Fluid*—Table I summarizes the results of twenty experiments in which the concentrations of reducing substances in cerebrospinal fluid and plasma from frogs are compared. The cerebrospinal fluid only once contained more than 81 per cent of the plasma reducing substances and its mean value was 66 per cent that of plasma. The blood collections were made so soon after the animals were removed from the cage that we believe the plasma figures represent basal values. In the last six experiments of Table I all of the cerebrospinal fluid which could be removed by moderate suction was collected from 8 to 30 minutes before a second collection was made. The concentration of this second specimen, though always higher than that of the first from the same animal, never rose to more than 81 per cent of the plasma value.

Table II summarizes the results of sixteen experiments in

TABLE I  
Concentration of Reducing Substances in Various Fluids from Frogs

Experiment No	Aqueous humor*	Plasma*		Cerebrospinal fluid*	Lymph*	Cerebrospinal fluid/plasma	Aqueous humor/plasma		Lymph/plasma
		Venous	Arterial				Arterial	Venous	
	mg per 100 cc fluid	mg per 100 cc fluid	mg per 100 cc fluid	mg per 100 cc fluid	mg per 100 cc fluid	per cent	per cent	per cent	per cent
1	12	16	19				63	75	
2	16	21						76	
3	15		25	11	23	44	60		92
4	26		25	18	28	72	104		112
5			30	17		57			
6	21	26						81	
7	22		32		30		69		94
8			33	23		70			
9	19		33				58		
10			35	20		57			
11	12		38†				32		
12			41	19		46			
13	30		42		38		71		91
14	36		42†				86		
15			42	28		67			
16	27		43		41		63		96
17			43	17		40			
18			47	43		91			
19	33		48	26	46	54	69		96
20			48‡	31		65			
21	39		50	29	48	58	78		96
22	36		52†				69		
23			54	44		81			
24			58‡	44	58	76			100
Mean...						63	69	77	97
Cerebrospinal fluid collected after preliminary drainage									
25			42	33		79			
26			48	33		69			
27			50	38		76			
28			53	43		81			
29			56	38		68			
30			63	48		76			
Mean						75			

\* Average of duplicate determinations.

† Frog not pithed.

‡ Blood obtained from web.

TABLE II

*Concentration of Inorganic Phosphate in Various Fluids from Frogs*

Experiment No	Phosphate P per 100 cc fluid				Cerebro-spinal fluid/plasma	Aqueous humor/plasma	Lymph/plasma
	Aqueous humor*	Blood plasma*	Cerebro-spinal fluid*	Lymph*			
	mg	mg	mg	mg	per cent	per cent	per cent
1	0 7	1 5	0 6		40	47	
2		1 7		1 3			77
3	0 8	1 9	0 7		37	42	
4	0 6	1 9	0 6		32	32	
5	1 0	1 9	0 9		47	53	
6	1 1	2 0	0 9		45	55	
7	1 3	2 4		2 4		54	100
8	1 4	2 6				54	
9	1 2	2 8		2 7		43	96
10	1 3	2 8				46	
11	1 3	2 8	1 1		39	46	
12	1 1	2 9	1 1		38	38	
13	1 4	3 1				45	
14	1 0	3 2				31	
15	1 3	3 4		3 3		38	97
16	1 4	3 5	0 8		23	40	
17	1 2	3 6	1 4		39	33	
18	1 6	3 6	1 6		44	44	
19	1 1	3 6				31	
20	1 4	3 7	1 5		41	38	
21		4 5		4 2			93
22	1 2	4 5				27	
23	1 8	4 7		4 7		38	100
24	1 0	5 1	0 9		18	20	
Mean					37	41	94
Preliminary subcutaneous injection of phosphate solution							
25	2 3	5 1	1 3		25	45	
26	1 6	6 3	0 8		13	25	
27	3 1	7 8	1 2		15	40	
28	2 4	9 2	0 9		10	26	
Mean					16	34	

\* Average of duplicate determinations.



which the inorganic phosphate content of cerebrospinal fluid and plasma from frogs are compared. In the first twelve experiments, where the plasma concentration ranged from 1.5 to 5.1 mg. per 100 cc., the cerebrospinal fluid phosphate never exceeded 1.6 mg. per 100 cc. and averaged 37 per cent of the plasma value. In the last four experiments, where the plasma concentration had been raised by the subcutaneous injection of a phosphate solution 1 to 2 hours before the experiment, the value of the cerebrospinal fluid remained below 1.4 mg. per 100 cc.

Table III summarizes the results of five experiments upon three depancreatized dogs in which the serum concentration of reducing

TABLE III  
*Concentration of Reducing Substances and Inorganic Phosphate in Various Fluids from Depancreatized Dogs*

Animal No	Time after operation	Reducing substances per 100 cc *			Phosphate P per 100 cc *		
		Aqueous humor	Serum	Cerebro-spinal fluid	Aqueous humor	Serum	Cerebro-spinal fluid
	hrs	mg	mg	mg	mg	mg	mg
1	48	306	351	225	1 2	3 9	1 1
1	72	364	372	220	1 4	3 9	0 9
2	72	460	520	292	1 6	5 4	1 3
3	48	296	352	220	1 7	3 0	1 2
3	72	356	420	220	2 1	3 3	1 1
3	79	368	436				

\* Average of duplicate determinations

substances lay between 351 and 520 mg. per 100 cc. In cerebrospinal fluid the reducing substances, though far above normal, never amounted to more than 64 per cent of the serum concentration. The inorganic phosphate of cerebrospinal fluid never amounted to more than 40 per cent of that of the serum, the concentration in both fluids remaining at a normal level.

*Aqueous Humor.* (a) *Frog*—The reducing substances of aqueous humor were compared with those of blood in fifteen experiments (Table I). In twelve instances, in which the comparison was with arterial blood, the mean aqueous humor value was 69 per cent of that of plasma. In three instances, in which the comparison was with venous blood, the mean aqueous humor value

was 77 per cent of that of plasma. Here, as in the experiments upon cerebrospinal fluid, we believe the plasma sugar was at basal level.

The inorganic phosphate concentration of aqueous humor was similarly compared with arterial blood plasma in twenty-six experiments (Table II). The average concentration of the aqueous humor was 40 per cent of that of blood. With respect then to both inorganic phosphate and reducing substances, the composition of frog aqueous humor is nearly identical with that of cerebrospinal fluid. In the four instances of Table II (Experiments 25 to 28), in which the blood level was raised by preliminary subcutaneous injection of phosphate solution, the aqueous humor concentration, unlike that of cerebrospinal fluid, increased to maintain its usual relationship to plasma.

In eight frogs we have evacuated one eye as completely as possible from two to five times within 60 minutes, and made phosphate analyses upon the series of aqueous humors thus obtained. The protein content of the successive specimens increased from approximately 2 to 50 per cent of the plasma specimen taken at the end of the experiment and they occasionally contained a few red blood corpuscles. The phosphate concentration of the specimens always rose, but, despite the pathological increase in capillary permeability<sup>3</sup> indicated by the protein changes, that of the final specimen averaged only 69 per cent of the plasma.

(b) *Fowls, Rabbits, Dogs, and Cats*—The composition of aqueous humor and that of blood from thirty-one animals have been compared in the experiments summarized in Table IV.

The mean concentration of reducing substances in the aqueous humor in fourteen experiments was 85 per cent of that in blood plasma. This difference between the two fluids is less marked than that which was found in frogs, and has less significance because the plasma concentrations were not certainly at basal

<sup>3</sup> In a few experiments we attempted to avoid a decrease in intraocular pressure by the injection of mineral oil simultaneously with the withdrawal of aqueous humor. The experiments were technically unsatisfactory and the specimens of aqueous humor continued to show abnormally large amounts of protein.

TABLE IV

*Reducing Substances, Inorganic Phosphate, Uric Acid, and Urea Concentrations in Aqueous Humor from Higher Animals*

Experiment No.	Animal	Reducing substances			Phosphate P			Uric acid†			Urea N		
		Plasma*	Aqueous*	Aqueous/plasma	Serum*	Aqueous*	Aqueous/serum	Serum*	Aqueous*	Aqueous/serum	Plasma*	Aqueous	Aqueous/plasma
		mg per 100 cc	mg per 100 cc	per cent	mg per 100 cc	mg per 100 cc	per cent	mg per 100 cc	mg per 100 cc	per cent	mg per 100 cc	mg per 100 cc	per cent
1	Fowl	226	216	81	2 3	0 9	39	4 83	4 0	83			
2	"	239	219	92		0 9		3 03	1 3	43			
3	"				2 4	1 0	42						
4	"				2 7	1 0	37						
5	Rabbit				2 9	1 7	59						
6	"	118	98	83	3 8	1 9	50						
7	"	142	148	104‡									
8	"	145	101	70	4 5	1 8	40						
9	"	163	165	101‡									
10	"	164	170	104‡									
11	"										15	8§	53
12	"										31 6	27 6	87
13	"										36 4	29 4	81
14	Dog	92	84	91‡									
15	"	97	77	79	3 1	0 9	29						
16	"	106	96	91‡	4 5	1 4	31						
17	"	107	81	76‡							18 7	15 6	83
18	"	123	101	82	3 3	1 1	33						
19	"	127	96	76‡									
20¶	"				6 4	2 0	31						
21	"										8 6	8 2	95
22	"										12 3	9 0	73
23**	"										13 6	6 7	49
24	"										15 1	9 9	66
25**	"										15 6	8 1	52
26	"										15 8	9 1	58
27	"				3 9	1 1	28				20 0	18 1	91
28¶	"										21 3	10 9	51
29¶	"										43 2	30 4	71
30	Cat	119	78	65	3 6	1 4	39						
31¶	"				8 0	1 6	20						
Mean				85			37			63			70

\* Average of duplicate determinations.

† Analyses by Dr J Bordley, 3rd.

‡ Analyses by Dr B. B Westfall with the Hagedorn-Jensen method.

§ Determined by the xanthohydrol method || Analyses by Dr. K. A. Elsom.

¶ Barbitol anesthesia.

\*\* Barbitol-morphine anesthesia.

level.<sup>4</sup> Some species difference apparently existed among the various animals; for, while three of the five rabbits showed an equilibrium between the two fluids, two rabbits, two fowls, six dogs, and one cat showed less reducing substances in aqueous humor than in plasma. In the seven experiments where both the Hagedorn-Jensen method and ultramicrotechnique were employed, the results of the two agreed.

The inorganic phosphate concentration of aqueous humor, determined in fourteen experiments, averaged only 37 per cent of that of serum. This difference between the two fluids was marked in all four species and was of the same order as that discovered in frogs.

Uric acid analyses were made upon the aqueous humor and serum of two fowls by Dr. James Bordley, 3rd. In both cases the aqueous humor value was well below that of the serum, though there were considerable differences in the degree of this deficiency.

The mean urea concentration of aqueous humor in ten dogs and three rabbits was only 70 per cent of that in plasma. With the exception of a single animal (Experiment 21) in which the plasma value was abnormally low, the difference between the two fluids from dogs was always considerable and beyond the limit of experimental error; it was somewhat more marked in the four animals which were anesthetized. In only two instances was the plasma value above 20 mg. per 100 cc. of urea nitrogen. We place less reliance upon the rabbit experiments because the two macroanalyses were made with 0.5 cc. of fluid, the plasma values were both above 30 mg. per 100 cc., and the third analysis was made by the xanthidrol method.

(c) *Depancreatized Dogs*—The results of six experiments are summarized in Table III. The mean value of the reducing substances in aqueous humor was 88 per cent of that in blood serum. They were far above normal and far above those of the cerebrospinal fluid specimens simultaneously obtained. In view of the rising level of the reducing substances in blood and the presumably slow formation of aqueous humor, the significance of this rather

<sup>4</sup> The rabbits of Experiments 7, 9, and 10 had access to food a short time before the fluids were collected. The high concentration of reducing substances in fowl blood is apparently normal for these animals (7).

small difference between the two fluids may be doubted. In terms of absolute amounts, however, it is still considerable and, like the difference between aqueous humor and plasma of normal dogs, would be nearly doubled were the relative water content of the two fluids taken into consideration.

The phosphate concentration of the aqueous humor in these experiments was only 44 per cent of that of serum, a relationship similar to that existing between the two fluids in normal dogs.

TABLE V

*Analyses of Aqueous Humor, Serum, and Serum Ultrafiltrate from Man*

Experiment No	Patient	Reducing substances				Inorganic phosphate				Uric acid†			Urea N‡			
		Serum*	Ultrafiltrate*	Aqueous*	Aqueous/ ultrafiltrate	Serum*	Ultrafiltrate*	Aqueous*	Aqueous/ ultrafiltrate	Serum*	Aqueous*	Aqueous/ serum	Serum*	Ultrafiltrate*	Aqueous*	Aqueous/ serum
		mg per 100 cc	mg per 100 cc	mg per 100 cc	per cent	mg P per 100 cc	mg P per 100 cc	mg P per 100 cc	per cent	mg per 100 cc	mg per 100 cc	per cent	mg per 100 cc	mg per 100 cc	mg per 100 cc	per cent
1	C. D.	99	85	54	64	3 13	1 17	55		2 2	1 1	50				
2	A. K.	111	100	60	60	4 14	2 2	1 50								
3	S. L.	108	105	69	66	3 83	8 19	50								
4	C. M.	113	109	56	51	2 83	0 20	67		5 1	4 5	88	17	17	6	35
Mean					60			55				69				

\* Average of duplicate determinations.

† Analyses by Dr. James Bordley, 3rd.

‡ Xanthidrol method.

(d) *Man*—Human aqueous humor, when compared with serum (Table V), exhibited deficiencies in phosphates, uric acid, and urea similar to those shown by the aqueous humor of frogs and higher animals. The mean reducing substance concentration of aqueous humor was 60 mg. per 100 cc.; of serum, 108. This difference between the two fluids cannot therefore be attributed to an abnormally high plasma concentration but must be regarded as a true deficiency.

## DISCUSSION

*Lymph*—The work of Starling (8) and of Landis (9) gives us every reason to believe that lymph derives from blood plasma by filtration through the capillary wall. The concentration of reducing substances and inorganic phosphates of the two fluids should therefore be identical if these substances exist in filtrable form. Arnold and Mendel (10) have already demonstrated the existence of such an equilibrium between lymph from the thoracic duct and blood serum in dogs. Our demonstration of a similar equilibrium in frogs is thus of only minor and confirmatory interest; it may have some slight theoretical advantage in that the lymph was collected from peripheral channels rather than from the thoracic duct. In view of work performed in this laboratory upon glomerular function, the demonstration of this equilibrium between frog lymph and plasma has the effect of confirming the reliability of the analytical methods for body fluids, and of providing a parallel for the similar equilibrium which has been demonstrated between glomerular fluid and plasma (2, 3).

*Cerebrospinal Fluid*—The early belief that cerebrospinal fluid was elaborated by secretory activity of the choroid plexus was based on histological demonstration of changes in the epithelial cells of this plexus (11), and on changes in cerebrospinal fluid pressure following the administration of drugs (12). Neither type of evidence is sufficiently unequivocal to determine whether the process in the choroid plexus is one of secretion or of filtration (13, 14). Direct chemical comparison between cerebrospinal fluid and blood should yield more decisive evidence and a large number of such comparisons for higher animals and man may be found in the literature. The most complete of these has been published by Fremont-Smith *et al.* (15). The numerous investigators agree that, though the total molecular concentration of the two fluids is similar,<sup>5</sup> there is marked disparity in the concentration of individual constituents. This has not led to the conclusion that

<sup>5</sup> The similarity in freezing point determinations is taken by Fremont-Smith and coworkers (16) as positive evidence that cerebrospinal fluid is a filtrate of plasma. If this were the case, both gastric fluid (17) and hepatic bile (18) would have to be accepted as filtrates.

cerebrospinal fluid is a product of secretion. There has been an inclination to explain the discrepancies on the basis of such physical determinants as Donnan's equilibrium and to conclude that the fluid is formed by simple filtration (19). These explanations are not altogether satisfactory, for they do not explain the disparities in urea, uric acid, and non-protein nitrogen which have been described by Cockrill (20) and others (15, 21, 22). They appear unable to explain the results of our determinations of reducing substances and phosphates.

This discrepancy in reducing substances between the cerebrospinal fluid and blood plasma of frogs (Table I) has been repeatedly demonstrated in higher animals and man. But, in the case of mammals it has been explained on these bases: (1) a portion of the plasma sugar (the non-fermentable reducing substances) was not in filtrable form, or (2) a portion of the plasma sugar was utilized by the brain tissue before the cerebrospinal fluid was collected. In our experiments these explanations do not apply. Frog plasma contains only about 5 mg. per 100 cc. of non-fermentable reducing substances and all of the plasma sugar must be in filtrable form since it all appears in an *in vitro* ultrafiltrate, in the web lymph, and in the glomerular urine (2) of intact frogs; it should, therefore, all appear in the cerebrospinal fluid if unmodified filtration through similar membranes occurs. The failure of cerebrospinal fluid sugar to approximate the plasma level after preliminary cisternal drainage (Table I, Experiments 25 to 30) argues against the sugar utilization hypothesis which is finally rendered untenable by the demonstration that this deficiency is maintained in depancreatized dogs (Table III) whose sugar utilization must be greatly diminished or abolished (23, 24).

The demonstration that frog cerebrospinal fluid contains but 40 per cent of the inorganic phosphate of blood plasma (Table II) again merely reflects what has been repeatedly found in mammals (15, 25-27). This discrepancy is the opposite of what would be expected if the Donnan equilibrium determined phosphate distribution. It cannot, in the case of the frog, be due to incomplete filtrability for all of the plasma phosphate appears in *in vitro* ultrafiltrates of frog plasma, and in the web lymph and glomerular urine of intact frogs (3). In view of the deficiency in depancreatized dogs (Table III), it cannot be explained as an incident

of carbohydrate metabolism. The cerebrospinal fluid phosphate remains low when the plasma concentration is raised by the subcutaneous injection of a phosphate solution (Table II, Experiments 25 to 28).

In view of these observations upon frogs and depancreatized dogs, we must conclude that the choroid plexus is partially impermeable to reducing substances and inorganic phosphate.

*Aqueous Humor*—The difficulty of collecting sufficient aqueous humor for analysis by the ordinary macrochemical methods probably explains the paucity of information concerning its composition. There have been no analyses of human aqueous humor excepting the sugar determinations of Ask (28). The most inclusive piece of work on the subject, regarded as expressing the "modern view" in this country and England, is that reported by Duke-Elder in 1927 (29). He collected a large pooled specimen of aqueous humor from a group of freshly killed horses and compared it with a specimen of "typical" horse serum obtained from the farm of the Medical Research Council. When allowance was made for minor discrepancies, he felt justified in concluding that aqueous humor was formed from plasma by simple dialysis. Specifically, and in so far as the four substances under present discussion are concerned, his analyses gave the following results.

	Sugar	Inorganic phosphate	Urea	Uric acid
	mg per 100 cc	mg P per 100 cc	mg per 100 cc.	mg per 100 cc
Aqueous humor	98	3 3	28	Not determined
Blood serum	91	3 0	27	

There is no further record in the literature of the urea or uric acid content of aqueous humor. Duke-Elder confirmed his view of the equilibrium in reducing substances by three experiments upon rabbits in which specimens of aqueous humor and venous and arterial blood were taken from each animal and their concentration determined by the Hagedorn-Jensen method. The average concentration of aqueous humor was 151 mg. per 100 cc., a figure slightly above that of venous blood, slightly below that of arterial blood. These results confirmed the experiments of Takahashi (30) upon



dogs. They were opposed by the experiments of de Haan and van Creveld (31) upon rabbits and of Adler (32) upon cats; these authors found less reducing substances in aqueous humor than in blood but believed that the discrepancy was due either to a portion of the sugar being "bound to protein" or to its utilization by the tissues of the eye. The phosphate equilibrium described by Duke-Elder was denied by Tron (33) in the course of a very careful study of the electrolytes of the aqueous humor and serum of oxen; he found only 60 per cent of the serum phosphate in the aqueous humor but his belief that a portion of the serum phosphate was "bound" brought the theoretical conclusion of his experiments into accord with that of Duke-Elder. There is then little existing information concerning the relation of aqueous humor to plasma with respect to the concentration of urea or uric acid. Determinations of reducing substances and phosphate have given discordant results, but those who have found deficiencies in these in aqueous humor have agreed that they are explicable on physical grounds.

80 experiments have been performed upon frogs, fowls, rabbits, dogs, cats, and men in which the composition of aqueous humor has been compared with that of blood from the same animal. The results of these experiments oppose the conclusion drawn by Duke-Elder in that they show (1) that the inorganic phosphate concentration of aqueous humor averages, in each species, less than 50 per cent of that in plasma, (2) that in rabbits, dogs, and man, the urea concentration averages only 68 per cent, and (3) that the uric acid concentration in fowls and man (four experiments) is only 66 per cent of that in plasma. In so far as reducing substances are concerned, three of our five experiments upon rabbits support Duke-Elder's belief that these substances are distributed equally between aqueous humor and blood; the results of twenty-seven other analyses principally upon frogs, dogs, and man, however, force us to conclude that in this respect too the aqueous humor shows a moderate deficiency when compared with blood. It appears fair to say that because of the micromethods which have been available, our experiments have certain advantages over those of previous investigators in the technique of fluid collections, in the variety of animals used, and in the number of experiments performed.

It seems impossible to reconcile these gross discrepancies in composition between aqueous humor and blood with known physical laws as they are at present understood and exemplified in the formation of filtrates such as lymph and glomerular fluid. The deficiency in reducing substances and phosphates cannot be attributed to their incomplete filtrability; from frog blood they have proved entirely filtrable both *in vivo* and *in vitro* (2, 3) and, so far as the evidence yielded by artificial membranes may be trusted, the same statement appears to hold in mammalian bloods (34, 35). In the human experiments our comparisons were made against the protein-free ultrafiltrates of serum. Nor can the deficiencies in either substance be explained as a direct or indirect consequence of carbohydrate utilization since both continued to occur in depancreatized dogs (Table III) whose sugar metabolism must have been either absent or greatly diminished (23, 24). The observation that the phosphate concentration of freshly formed aqueous humor remains below that of plasma also indicates that this deficiency is not concerned with the carbohydrate utilization of the eye, and its distribution between the two fluids is not explicable on the basis of a Donnan equilibrium. Since uric acid has been shown to be completely filtrable through parlodion membranes and through the glomeruli of frogs and snakes (4), the only living membranes in which its filtrability has been directly investigated, it is difficult to explain its partial absence from aqueous humor on purely physical grounds. We know of no physical explanation which would account for the deficiency in urea which aqueous humor displays in comparison with plasma; it is an eminently filtrable substance and is present in the glomerular urine of frogs in concentration identical with that of plasma (36).

#### SUMMARY

1. Reducing substances and inorganic phosphates are present in lymph from the frog's web in approximately the same concentration as in blood plasma. The agreement of this finding with established belief in the filtration theory of lymph formation is regarded as additional evidence of the validity of the ultramicro-analytical methods used in the greater part of this work.

2. The concentration of reducing substances in cerebrospinal fluid from frogs is about 30 per cent less than that in plasma; of

inorganic phosphate, 60 per cent less. Similar differences exist between these fluids taken from completely depancreatized dogs.

3. The aqueous humor of frogs, in comparison with plasma, shows the same deficiency in reducing substances and inorganic phosphates as the cerebrospinal fluid. In higher animals and man, the deficiency of phosphates was uniformly present; that of reducing substances, though less marked and less constant, was usually recognizable. The urea concentration in aqueous humor from rabbits, dogs, and man is on the average 30 per cent less than in plasma. The concentration of uric acid in aqueous humor from fowls and man is about 34 per cent less than that in plasma.

4. Reasons are given for the belief that neither incomplete filtrability nor utilization of carbohydrates by tissues of the brain or eye can explain these discrepancies. The conclusion is drawn that the choroidal and the ciliary epithelium exhibit selective qualities not possessed by capillary endothelium or by the glomerular membrane, and that neither cerebrospinal fluid nor aqueous humor is formed by simple processes of filtration or dialysis.

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## A SIMPLE METHOD FOR THE DETECTION AND ESTIMATION OF *l*-XYLOKETOSE IN URINE

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In nine cases of pentosuria the urinary sugar was proved to be *l*-xyloketose by identification of its osazone. We discovered that in each of these pentosurics the urine reduced Benedict's sugar reagent (1) in a few hours without the application of heat, thus exhibiting a more active reducing power than does the urine of a diabetic, even when it contains a high percentage of glucose. This property was undoubtedly due to the pentose present, for after such a reduction in the cold, the pentose had completely disappeared. These findings have been developed into a method for the detection and estimation of *l*-xyloketose in urine.

In order to determine the most favorable conditions for a useful test we investigated the effect of temperature on the time required for reduction by the urine of pentosurics and diabetics, and by solutions of various sugars, including a purified syrup of xyloketose.

The procedure was as follows: 1 cc. of urine or of sugar solution was added to 5 cc. of Benedict's qualitative sugar reagent in a test-tube, thoroughly mixed, and the tube was then placed in a water bath at a constant temperature. We determined the reduction at room temperature (20–30°), 40°, 50°, and 60°. The time required for the appearance of a definite yellow precipitate was noted. Some slight difficulties may be encountered in determining the onset of reduction, due to the formation of a phosphate precipitate; however, if the tube is observed by reflected light, the yellow precipitate of cuprous hydroxide is easily distinguished from the white or grayish phosphate.

The sugars and the diabetic urines were diluted by the addition of normal urine to a concentration that would more nearly approach conditions in natural pentosuria. We had found previ-

ously that, when urine was used as a diluent rather than water, the reduction reaction of sugars was retarded.

TABLE I  
*Time Required for Onset of Reduction at Different Temperatures*

Substance analyzed	Subject	Reducing bodies as glucose	Room temper- ature	40°	50°	60°
		per cent	min	min	min	min.
Urine, pentosuria	L. B.	0 3	73	11	4 5	2
" "	J. L.	0 35	*	27	8	3 5
" "	"	0 30	185	25	8	4
" "	A. G.	0 2	140	15	6	2
" "	B. B.	0 12	160	33	7	5
" "	N. L.		185		5	2
" "	B. L.	0 8	75	13	4	2
" "	C. J.		90		5	
" " .....	M. C.	0 14	180	22	7	3
" " .. . . .	S. J.	0 38	110	12	4 5	1 7
" levulosuria		0 3†	*	60	24	7
" diabetes		0 5§	*	>390	150	43
" "		0 5§	*	>390	112	30
" "		0 5§	*	>390	120	30
" "		0 5§	*	>390	140	28
" "		0 5§	*	>390	110	30
" "		0 5§	*	>390	110	27
" "		0 3	*	>390	240	70
" "		0 4	*	>390	110	25
" " ..		0 5§	*	>390	135	40
" " ..		0 5§	*	>390	130	30
" "		0 5§	*	>390	115	27
l-Xyloketose		0 3	35	6	1 5	<0 2
l-Xylose		0 3	*	182	43	18
dl-Arabinose		0 3	*	450	58	27
Glucose		0 3	*	550	68	28
d-Fructose		0 3	*	47	20	8
Lactose ..		0 3	*		88	39

We are indebted to Sydenham Hospital for the urine of case B. L., and to Dr. S. Silver for the specimen of levulosuric urine.

\* No reduction within 24 hours.

† Diluted from a concentration of 1.58 per cent. § Diluted.

The results of the experiment are given in Table I. They show the relatively short time which xyloketose requires to reduce the copper solution. The urines of all the pentosurics reduced it in less than 10 minutes between the temperatures of 50–60°.

At 40° most of the pentosuric urines reacted within 15 minutes. At room temperature all of them (with a single exception, which will be discussed later) reduced the Benedict's solution within 3 hours. The diabetic urines, on the other hand, showed no reduction after many more hours, usually not at all, while all the common sugars (except *d*-fructose) remained completely inactive for 24 hours.

Fructose, which, like xyloketose, is a ketose, reduces quickly at temperatures of 50–60°, but much more slowly than xyloketose below 40°. Cases of fructosuria (levulosuria) have been reported in the literature. They occur very rarely but can easily be differentiated from pentosuria, since the fructose is fermented by yeast, does not give a Bial's test, forms a glucosazone with phenylhydrazine, and gives a Seliwanoff reaction.

As a result of this study we have used the following test for the quick identification of xyloketose in urine.

*Qualitative Test*—1 cc. of urine and 5 cc. of Benedict's qualitative sugar reagent are mixed in a test-tube and placed in a bath at 55°. The tube is observed at the end of 10 minutes. The appearance of a yellow precipitate indicates that the urine contains *l*-xyloketose or *d*-fructose.

Margolis (2) has observed that pyramidon (amidopyrine) causes an increase in xyloketose excretion in a pentosuric. When the above test was applied to the urine of pentosurics after pyramidon medication, the reaction was greatly intensified, reduction starting immediately. At room temperature it occurred within an hour. Instances of the effect of pyramidon on the excretion of reducing substance in several cases of pentosuria are shown in Table II. It will be seen that the amount of reducing substance increases from 2 or 3 gm. to as much as 14 gm. while the concentration increases 4-fold.

That the increase was one of xyloketose<sup>1</sup> was shown by the following quantitative determination.

<sup>1</sup> The possibility of the increase in reducing bodies after pyramidon being due to free glucuronic acid is excluded, since glucuronic acid does not reduce in the cold, according to Ehrlich (3), nor at 55°, as determined by ourselves. Greenwald, as quoted by Margolis, showed the increase after pyramidon medication in Margolis' case of pentosuria to be pentose, by gravimetric estimation of the osazone. He found the yield, however, was less than 50 per cent of the theoretical.



**Quantitative Determination**—The amount of urine required to reduce 2 cc. of Benedict's quantitative sugar reagent (4) at 55° is estimated as follows: First determine the volume of urine ( $v_1$ ) required to complete the reaction at the boiling point, then take a series of test-tubes, 12 mm. in diameter, containing 2 cc. of Benedict's solution, 0.5 to 1.0 gm. of anhydrous sodium carbonate, and measured amounts of urine; start with volume  $v_1$  and increase the amount in successive tubes by 0.1 to 0.2 cc. Shake the tubes and immerse them in a bath at 55° for 10 minutes. Observe which

TABLE II  
*Influence of Pyramidon on Pentose Excretion in Pentosuria*

Pentosuric patient	Reducing substance in 24 hrs (as xyloketose)			
	Normal		After pyramidon	
	gm.	per cent	gm	per cent
L. B.	3 3	0 25	14 0	1 02
J. L.	3 2	0 23	6 9	0 48
A. G.	1 9	0 16	2 8*	0 23
B. B.		0 10	3 0	0 34

\* Single dose

tube contains the least amount of urine sufficient for complete reduction and calculate for xyloketose as follows:

$$\text{Per cent xyloketose} = 0.0033/v_2 \times 100 \quad (1)$$

$$\text{Gm. xyloketose in 24 hrs} = 0.0033/v_2 \times V \quad (2)$$

where 0.0033 = gm. of xyloketose equivalent to 2 cc. of Benedict's solution,<sup>2</sup>  $v_2$  = cc. of urine required for reduction at 55°,  $V$  = cc. of urine in 24 hours. If greater accuracy is desired the intervals may be determined by using Equation 2. Time may be saved by making a preliminary computation. Since ordinarily not more than 1 gm. of reducing substances is present in urine, the following equation will give an approximate value for  $v_2$  and so determine the range in which to work:  $v_2 = 0.0033V/(R - 1)$ , where  $R$  = gm. of total reducing substance in 24 hours.

Table III shows the xyloketose and undetermined reducing substances in the urine of patient A. G. on 4 consecutive days on

<sup>2</sup> Greenwald (5) gives 1.22 mg. as the glucose equivalent of 1 mg. of xyloketose by the Benedict method. We have used this figure throughout.

regular hospital diet, and the influence of one dose of pyramidon on this patient.

*Applications of Method*—The method which we have described is applicable in distinguishing a xyloketosuria from a possible arabinosuria (*dl*-arabinose added to normal urine does not react) and in detecting xyloketose in the presence of glucose. A diabetic urine containing 0.05 per cent of added xyloketose reacted positively to the test, whereas the urine containing 4 per cent glucose was negative.

TABLE III

*Proportion of l-Xyloketose to Total Reducing Substance Excreted in 24 Hours*

Total reducing substance	l-Xyloketose		Undetermined reducing substance (as xyloketose)	
gm.	gm.	per cent	gm.	per cent
2 03	1 64	81	0 39	19
1 81	1 45	80	0 36	20
1 70	1 39	82	0 31	18
1 97	1 45	74	0 52	26
• 2 80*	2 16	77	0 64	23

\* One dose of pyramidon.

The method has been used for the quantitative estimation of xyloketose in concentrates, as well as in the presence of glucuronic acid and xylose.

#### DISCUSSION

Garrod (6) states that several investigators have noticed delayed reduction by pentosuric urine, but that Bial observed that reduction occurs before the boiling point is reached.

Medes (7) has investigated a case of tyrosinosis in which an unusual reduction occurred when molybdic acid reagent of the Fiske and Subbarow phosphorus method was added to the urine of a patient with myasthenia gravis. This urine also reduced alkaline copper solutions, but more slowly than does glucose.

While making a creatinine determination by the Folin method (8) on the urine of a pentosuric containing a high concentration of xyloketose, it was noted that not only did the creatinine figure appear too high, but also that the intensity of the color gradually increased on standing. Further investigation showed that xyloke-

tose behaves in a similar manner. This phenomenon is to be studied more in detail.

We have noticed that with the same concentration of xyloketose in urine the time required for reduction is not always the same. Other substances present have a retarding effect, which is less conspicuous when the xyloketose is more concentrated. One case of pentosuria, J. L., excreting 2.9 gm. of xyloketose daily, excretes a urine which sometimes—especially after it has been standing—gives no reduction at temperatures of 20–30°, although at 55° the reduction always occurs within the time limit of the test. On intensification of the test by pyramidon administration, as previously described, reduction was obtained at the lower temperatures. Various different urine specimens of each of the other pentosuric cases were tested, all reacting between 20–30°. The exception may be due to the presence of a high concentration of retarding substances in that particular urine. However, the reducing power of some xyloketose added to this urine was not inhibited.

#### SUMMARY

1. A simple test for the detection of *l*-xyloketose in urine has been developed from a study of nine cases of pentosuria where corroborative chemical tests had proved the sugar to be *l*-xyloketose. Where very small amounts of pentose are suspected or where doubt exists, an increased elimination of pentose can be brought about by pyramidon medication.

2. This method was used for the quantitative determination of xyloketose in the presence of other reducing substances.

3. In the determination of creatinine by the Folin method, pentosuric urines give a color which intensifies on standing. Preliminary investigation indicates that the increased color is due to xyloketose.

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## SOME OBSERVATIONS ON BLOOD PHOSPHATE

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### INTRODUCTION

As recommended by Fiske and Subbarow (1), a 10 per cent solution of trichloroacetic acid is safe to use as a protein precipitant for the determination of inorganic phosphate in blood. Although the use of such a high concentration has some disadvantages, it appears, on the other hand, that this drastic treatment is necessary in order to obtain rapidly a clear, protein-free filtrate.

Greenwald (2) had previously pointed out that an acid-soluble ester of phosphoric acid occurs in blood. He isolated this ester which is extremely resistant to acid hydrolysis and identified it as diphospho-*l*-glyceric acid. Prior to his investigation, Zucker and Gutman (3) had submitted the trichloroacetic acid filtrate of blood to acid hydrolysis. They had reported that while one portion of the organic phosphate was readily split off, another portion was very resistant to acids. They called it "non-hydrolyzable." Kay and Robison (4) investigated Zucker and Gutman's findings and showed that upon prolonged hydrolysis all the organic phosphates of blood are hydrolyzable with acids; moreover, they found that one portion could be acted upon by bone phosphatase, while the acid-resistant ester could not be. Later, Goodwin and Robison (5) investigated the nature of the easily hydrolyzable acid-soluble organic phosphate fraction and suggested that it consisted of two esters of the nature of hexosephosphate.

In this paper are recorded (a) some observations on the determination of the inorganic phosphate of whole blood; (b) the blood phosphate of fasting and fed rabbits; and (c) a brief investigation of the rate of hydrolysis of the easily hydrolyzable phosphoric ester.

*Determination of Blood Phosphate*—Rabbit blood was used throughout this investigation. As suggested by Fiske and Subbarow, for every 4 cc. of 10 per cent trichloroacetic acid in a small flask 1 cc. of whole blood was slowly introduced and the mixture thoroughly shaken. The precipitated blood was filtered within 5 minutes. In this experiment, the results of which are found in Table I, 3 cc. of blood were used in each case in order that enough filtrate could be collected to make two estimations of the inorganic phosphate. One sample was immediately analyzed; the other was corked and allowed to stand for 24 hours at room temperature

TABLE I

*Inorganic Phosphate of Rabbit Blood*

Inorganic phosphate was determined immediately and after allowing the filtrate to stand 24 hours at room temperature.

Rabbit No	Determination	P per 100 cc blood	Rabbit No	Determination	P per 100 cc blood
		<i>mg</i>			<i>mg</i>
3	Immediately	4 65	8	Immediately	5 35
	After 24 hrs.	5 35		After 24 hrs.	6 15
4	Immediately	5 65	9	Immediately	6 65
	After 24 hrs.	6 40		After 24 hrs.	7 90
5	Immediately	6 15	10	Immediately	6 65
	After 24 hrs.	7 15		After 24 hrs.	7 85
6	Immediately	5 35	11	Immediately	6 15
	After 24 hrs.	6 30		After 24 hrs.	7 60
7	Immediately	3 65			
	After 24 hrs.	4 45			

before analysis. It is apparent that the 24 hour samples yielded higher values for inorganic phosphate than those immediately analyzed. This increase is undoubtedly beyond any experimental error. Inorganic phosphate was also determined on blood samples removed from the animals at 1.5 and 3 hour intervals. No changes in inorganic phosphate were noted.

The effect of various concentrations of trichloroacetic acid on whole blood precipitation was studied. Concentrations of 3, 4, 5, and 6 per cent were found to be unsatisfactory, for blood proteins were not completely removed. This confirms the previous findings of Fiske and Subbarow. Although the employment of 10

per cent trichloroacetic acid may be considered drastic, yet good results could easily be obtained provided both filtration and analysis were performed immediately. Keeping the filtrates alone or the filtrates including the precipitated blood in an ice box, apparently does not prevent hydrolysis of the labile phosphoric ester.

*Blood Phosphate of Fasting and Fed Rabbits*—Though the primary object of this investigation was not a study of the changes that occur in blood phosphate of fed and fasted animals, yet it was observed that the inorganic phosphate of the blood of rabbits after a 24 hour fast was considerably higher than that of the fed animal.

TABLE II

*Inorganic Phosphate of Blood of Fasting and of Fed Rabbits, Showing Effect of 10 Per Cent Concentration of Trichloroacetic Acid on Blood Phosphate Determination*

Rabbit No.		P per 100 cc blood	
		Immediately	After 24 hrs
		mg	mg
1	Fed	3 08	3 98
	24 hr. fast	8 00	8 95
2	Fed	3 48	4 24
	24 hr. fast	7 60	7 94
3	Fed	3 22	3 98
	24 hr. fast	6 95	7 40
4	Fed	3 94	3 94
5	"	3 85	4 90

This interesting observation was noted in all of our experimental animals. Some of the data illustrating this observation are found in Table II.

*Hydrolysis of Blood Phosphoric Acid Ester*—According to Kay (6) it is essential that the precipitate of blood plasma should not remain in contact with the acid for more than a few minutes; otherwise the hydrolysis of lipids takes place, yielding a phosphoric ester. Although Kay observed no changes in the inorganic phosphate when his filtrates stood for 168 hours, it was considered advisable, since whole blood was used, to avoid the presence of lipids.

In accordance with Bloor's technique the extraction of lipids was undertaken before the precipitation of blood proteins by trichloroacetic acid. Inorganic phosphate was then determined quantitatively on the lipid-free precipitate. Since the extraction of phospholipids with alcohol-ether had no effect on the labile phosphate, it is therefore probable that this labile phosphate is neither a lipid nor a lipid-like substance.

*Acid Hydrolysis*—When samples of the acid filtrates were treated with sulfuric acid (1 cc. of 5 N sulfuric acid to every 5 cc. of the trichloroacetic acid filtrate of blood) and hydrolyzed for 2 hours, hydrolysis of an organic ester of phosphoric acid took place. Apparently this hydrolyzable ester is not Greenwald's diphospho-*l*-glyceric acid, since according to Greenwald (2) its hydrolysis in 5 per cent sulfuric acid requires several days. Whether or not lipids were first extracted from the filtrates, the amount of inorganic phosphate split off was found to be the same in each sample after hydrolysis. In this we confirm the earlier findings of Zucker and Gutman (3).

The above observation led to the investigation of the rate of hydrolysis of this phosphoric ester.

*Procedure*—20 cc. of blood were precipitated with 10 per cent trichloroacetic acid and the filtrate collected. In each of a series of graduated test-tubes, 5 cc. of the clear filtrate were introduced along with 0.5 cc. of 5 N sulfuric acid. The tubes were then fitted with loose glass stoppers and at once immersed in a boiling water bath. 3 minutes were allowed to pass before the first sample was removed. This sample was used for the determination of the initial amounts of the inorganic phosphate and that fraction of the phosphoric ester that hydrolyzed before the temperature became constant. As soon as each tube was removed, it was immersed in an ice water bath and neutralized with approximately 2 N sodium hydroxide<sup>1</sup>. In determining the inorganic phosphate the reagent containing ammonium molybdate in 5 N sulfuric acid was used. The results of this experiment are found in Table III.

<sup>1</sup> Neutralization was previously performed on samples containing the same amount of sulfuric acid and trichloroacetic acid filtrate as was used for the experiment in question. Phenolphthalein was used as indicator. 2.2 cc. of approximately 2 N sodium hydroxide were required to neutralize a sample of 5 cc. of the acid filtrate in 0.5 cc. of 5 N sulfuric acid.

It is obvious, as can be seen from the data presented, that the rate of hydrolysis of that fraction of the acid-soluble phosphoric ester which hydrolyzes in the acidity above mentioned follows the monomolecular reaction.

TABLE III

*Rate of Hydrolysis of Phosphoric Acid Ester of Trichloroacetic Acid Filtrate of Rabbit Blood*

Showing the rate of hydrolysis of the easily hydrolyzable phosphoric acid ester of the acid-soluble phosphate of rabbit blood.  $K$  was calculated according to the monomolecular equation. Inorganic phosphate split off is expressed in mg. of P per 100 cc. of blood.

Time	Inorganic P	$a - x$	$K^*$
min.	mg.	mg.	
0 (Initial)	7 30	0 00	
5	8 30	2 60	0 064
10	9 15	1 85	0 064
20	9.95	0 95	0 066
30	10 40	0 50	0 066
40	10 60	0 30	0 062
50	10 90		
75	10 90		

$$* K = \frac{1}{t} \ln \frac{a}{a - x}.$$

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## STUDIES ON KETOSIS

### III. THE COMPARATIVE GLYCOGEN FORMATION AND RETENTION AFTER THE ADMINISTRATION OF GLUCOSE, GALACTOSE, AND LACTOSE\*

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#### INTRODUCTION

Galactose has been found to exert a superior ketolytic action over that of glucose by Deuel, Gulick, and Butts (1). In view of the fact that the antiketogenic effect was still pronounced on the 3rd day after the administration of a single dose of 75 gm. of this sugar, we naturally looked first to a variability in glycogen response as an explanation for this difference between these sugars.

Most authors are in general agreement that both galactose and lactose are far less efficient glycogen formers than is glucose. Murchhauser (2), using dogs as experimental subjects, found that the increase in liver glycogen after the feeding of various sugars was only 13 and 6 per cent in the case of galactose and lactose of what it was after glucose. The experiments of Haffmans (3) were similar. In both cases glucose was reported as having the greatest efficiency as a glycogen former of any of the carbohydrates. Cori

\* A preliminary report of some of these data has been made in *Proc. Soc. Exp. Biol. and Med.*, **30**, 24 (1932-33). Some of these results were made possible through the generosity of the Committee on Scientific Research, American Medical Association (Grant 214).

The experiments on the glycogen formation in dogs are included in a thesis on the chemical study of various glycogens presented by Paul W. Jewel in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate School of the University of Southern California.

(4) found that the total sugar retained as glycogen in the liver of that absorbed 4 hours after its administration to rats previously fasted for 48 hours was: glucose, 17 per cent, fructose, 39 per cent, and galactose, 5 per cent. Galactose actually is more rapidly absorbed from the gastrointestinal tract than is glucose (Cori (5)). The absorption of lactose, particularly in the case of adult dogs, is known to be slow, so this in part may be the reason for the poor effect with this sugar noted above.

However, the glycogen formation has been compared by these investigators after the *administration* of like amounts of these sugars rather than after the *retention* of similar quantities. Obviously, this is an important consideration in Cori's experiments (4) in which as much as 60 per cent of the administered galactose was excreted in the urine and was no longer available as a possible source of glycogen. When this sugar is ingested by man, the loss in the urine is relatively not so great. In the extensive experiments of Harding and Moberley (6), as well as those already referred to by the authors (1), it amounts to less than 4 per cent after doses as large as 75 gm.

Moreover, investigators (2-4) have not studied the effects at periods longer than 8 hours after galactose feeding nor has any extensive experimental work been carried out on the comparative response of the muscle glycogen. Lastly, any investigation of the comparative behavior of the sugars should involve a determination of the glycogen after the continual feeding of the animals for several days on diets high in one or the other constituent sugar. This seems a more physiological procedure than a single administration of an exceedingly large dose of the sugar.

The present paper reports the glycogen formation in dogs and rats after the retention of like quantities of galactose and glucose as well as that at various periods of fasting following the administration of diets high in glucose, galactose, lactose, or galactose and glucose. This last method has been by far the most satisfactory for the demonstration of the superior glycogenetic action of galactose and lactose and the results have been exceedingly consistent.

#### EXPERIMENTAL

*General Methods*—Amytal was used as an anesthetic in all of the experiments, inasmuch as it causes no considerable glycogen-

olysis, since one obtains a constant blood sugar level after its administration (7). The tissues on which glycogen was to be determined were quickly removed immediately after anesthesia was complete and frozen in CO<sub>2</sub>-ether mixture or in liquid oxygen (Series I). They were weighed while still frozen, put in boiling 40 per cent KOH solution, and digested in a boiling water bath for 3 hours. Glycogen was determined by a combination of the Pflüger method and that of Shaffer and Hartmann.

The whole livers were used in the experiments with rats. In the case of those on dogs, samples of suitable size were removed from each lobe and the average value used for the glycogen content of the entire organ. The muscles were removed from the posterior portion of the right rear leg of the rat for the glycogen determinations. In the dog experiments the gastrocnemius muscle was frozen *in situ*.

Blood sugar was determined by the Shaffer-Hartmann method. The estimation of urine sugar in the dog experiments was carried out by the Bertrand procedure, while in the tests on rats the Shaffer-Hartmann technique was used after precipitation of the interfering substances with Lloyd's reagent. Surface area on the dogs and rats was calculated by the Meeh formula.

*Experiments on Dogs*—It seemed advisable to carry out experiments on these animals in view of the fact that the results of Murchhauser (2) and of Haffmans (3) had shown galactose to have only a feeble glycogenetic ability when compared with glucose. It is possible that the reason why these investigators did not find any appreciable glycogen formation after galactose may be ascribed to their failure to compare animals in which identical amounts of these monosaccharides had been retained rather than fed. Moreover, it may be that the glycogenetic ability of galactose only becomes manifest after a period longer than 8 hours following its administration. It should be noted that Deuel and Chambers (8) have shown that galactose exerts as great a nitrogen-sparing action in the phlorhizinized dog as does glucose.

Female dogs which had previously been fasted for 6 days were used as subjects. Two animals as nearly similar in size and in breed as were obtainable were used in the experiments, one of which received galactose and the second of which was fed an amount of glucose corresponding with the galactose retained.

TABLE I

*Comparative Glycogen Content of Liver and Muscle of Female Dogs at Various Periods after Galactose or Glucose Administered on 6th Fast Day*

Time after carbohydrate feeding	Galactose-fed dogs					Glucose fed dogs		Glycogen			
	Dog No	Body weight	Galactose			Dog No	Body weight	Liver		Muscle	
			Fed	Excreted	Retained			After galactose	After glucose	After galactose	After glucose
hrs		kg	gm	gm	per cent		kg	per cent	per cent	per cent	per cent
6	17	10 0	39 2	13 3	66	22	6 7	3 78	3 80	0 40	0 43
	16	14 4	50	25 4	49	34	11 9	2 93	4 08	0 28	0 56
Average								3 36	3 94	0 34	0 49
12	18	10 6	40 7	22 5	45	26	10 0	5 77	2 96	0 48	0 62
	21	14 4	49 8	23 2	53	35	12 6	4 70	4 15	0 32	0 51
	44	13 3	47 4	19 9	58	53	12 5	4 82	2 90	0 35	0 40
Average								5 10	3 34	0 38	0 51
18	29	16 7	55 2	28 7	48	31	19 8	3 80	2 78	0 54	0 42
24	24	7 0	31 0	14 4	54	28	7 5	3 22	2 03	0 16	0 46
	47	5 6	26 5	15 7	41	49	4 8	2 67	1 76	0 63	0 65
	50	8 1	34 2	17 4	49	52	9 6	3 25	1 98	0 64	0 75
Average								3 05	1 92	0 48	0 62
48	51	6 5	29 4	16 8	43	54	8 0	3 07	1 32	0 46	0 52
	39	7 7	33 0	17 5	47	41	6 6	1 82	0 82	0 23	0 38
	38	8 7	36 2	18 4	49	40	8 0	2 68	3 07	0 22	0 39
Average								2 52	1 73	0 30	0 43
72	57	13 6	48 1	15 3	68	63	15 9	2 21	1 55	0 32	0 35
	62	17 3	40 3	10 8	73	70*	11 8	2 49	0 04	0 57	0 10
Average								2 35	0 80	0 45	0 22

\* Pregnant

Galactose was fed in an amount of 65 gm per sq m of body surface and approximately 50 per cent was retained. In order to determine whether sufficient glycogenolysis had occurred as a result of

the anesthetic or the operation to lessen the glycogen content of the tissues, the blood sugar was compared before the anesthetic was administered and after the completion of the operation. A uniform level was taken as an indication that the amount of glycogenolysis was negligible.

Table I gives the values on the animals killed 6, 12, 18, 24, 48, and 72 hours after the administration of galactose or glucose.

With the exception of the animals killed 6 hours after the administration of the sugars, the liver glycogen is consistently higher in the galactose-fed animals. Moreover, except for the 48 hour group, there is no overlapping of the values. The maximum level for the glucose-fed dogs was reached after 6 hours, while that for the galactose-fed ones was obtained 12 hours after its feeding. The average of 5.10 per cent for the liver glycogen of the dogs fed galactose 12 hours previously represents a value higher than will usually be found with well fed animals. In all the experiments included in Table I as well as in Table II, there have been no significant changes in the blood sugar level from the period before amytal was administered to the one following the completion of the operation just prior to the removal of the liver samples and muscle. This is in harmony with the earlier investigations which showed that this hypnotic was without appreciable effect on the level of blood sugar (9). In only one case have we found any significant alteration and that experiment has been deleted.

There can be little doubt that the liver glycogen is considerably increased in the animals which received the sugar over that which it would have been without it. The values for the fasting controls, which indicate the level at the time when the carbohydrates were fed, are given in Table II.

The average liver and muscle glycogen of 0.45 and 0.31 per cent respectively for the fasting controls is lower than that found on any of our sugar-fed animals during the first 24 hour period. Although the controls were males and the animals which received the sugar were females, we know that the females would tend to have still lower glycogen values if fasted the same length of time (10).

However, because of the small number of satisfactory experiments which we had succeeded in completing and because of our lack of success in obtaining dogs of uniform breed and size, we abandoned further study of this problem with these animals. The

variability in the animals in the experiments we have reported is best shown in the lack of uniformity in muscle glycogen which must depend to a considerable extent on the amount of exercise and development of the muscle and probably the age as well as on the nutritional condition. We feel that the limited number of experiments proves definitely that galactose is extremely efficient as a glycogen former in dogs and that it probably excels glucose in this respect. Moreover, our conclusions are based on the results from fourteen dogs receiving each sugar, while the negative results of Murchhauser (2) involve a comparison only of a single dog on each sugar and these animals varied considerably from one another

TABLE II  
*Liver and Muscle Glycogen of Male Dogs Fasted 6 Days*

Dog No	Breed	Body weight	Glycogen	
			Liver	Muscle
		<i>kg</i>	<i>per cent</i>	<i>per cent</i>
19	Fox-terrier	6 4	0 19	0 14
20	Mastiff	11 4	0 86	0 44
23	Wolfhound	11 3	0 24	0 40
37	Police	10 5	1 03	0 40
42	Collie	18 0	0 03	0 24
43	Foxhound	7 0	0 16	0 44
46	Pekingese	4 0	0 61	0 12
Average . .			0 45	0 31

in weight. In addition to the experiments reported in Table I we have carried out six tests on dogs after the administration of galactose which are not reported here because no comparative experiment on glucose was available. The liver glycogen was uniformly much higher than the control level and these experiments offer additional evidence for glycogenesis after galactose.

In a further study of the glycogenetic action of galactose, we have used rats as experimental animals. It was felt that because of the previous negative evidence in the literature, only by a large number of tests could we obtain sufficient evidence to prove or disprove a possible superior glycogenetic action of galactose. Moreover, with rats we could obtain greater uniformity as to age and strain and by so doing obtain consistent results on the muscle

glycogen. Cori's experiments (4), in which galactose was found to play an unimportant rôle in glycogen formation, were carried out on rats.

*Experiments on Rats with a Single Administration of Sugars*—In this series of investigations, galactose was fed by stomach tube to rats previously fasted for 48 hours in a dose of 5 mg. per sq. cm. of body surface in 50 per cent solution and the animals were killed at intervals of 3, 5, 7, 9, and 24 hours thereafter. Control animals,

TABLE III  
*Average Liver and Muscle Glycogen and Muscle Sugar of Rats Previously Fasted 48 Hours*

Time after sugar	Type of experiment	After galactose administration				After glucose administration			
		No of animals	Glycogen		Muscle sugar	No of animals	Glycogen		Muscle sugar
			Liver	Muscle			Liver	Muscle	
hrs			per cent	per cent	mg per cent		per cent	per cent	mg per cent
3	Sugar fed	3	0 71			3	1 61	0 26	
	Control	3	0 18			3	0 04	0 13	
5	Sugar fed	6	0 67	0 19	87 7	4	1 06	0 24	38 1
	Control	6	0 09	0 14	30 9	4	0 13	0 16	31 4
7	Sugar fed	6	0 59	0 21	66 6	4	1 07	0 25	54 4
	Control	6	0 09	0 14	26 5	4	0 03	0 15	37 6
9	Sugar fed	4	0 70	0 23	61 1	4	1 32	0 24	56 3
	Control	4	0 06	0 14	35 0	4	0 00	0 16	41 5
24	Sugar fed	4	0 88	0 22	64 1	4	1 03	0 20	37 2
	Control	3	0 10	0 14	52 1	4	0 08	0 16	29 7

which were usually litter mates, were given water instead of glucose solution and killed at the same time. Another group of animals—also litter mates—was given glucose in proportion to their surface area in an amount equivalent to the galactose absorbed and retained during each period. This was determined by subtracting from the quantity administered that unabsorbed in the gastrointestinal tract and that excreted in the urine. The former was ascertained by an analysis of the extract of the entire gastrointestinal tract removed at the time the animal was killed. The validity of our technique was demonstrated by the recovery of slightly over 90 per cent of the glucose administered from the gastrointestinal tracts of animals killed within a few minutes after



the administration of the sugar solution. For the determination of the sugar, the minced gastrointestinal tract was extracted several times with water, the proteins were precipitated with tungstic acid, and the supernatant liquid following centrifugation after each extraction, made up to volume. Muscle sugar was determined on the tungstic acid filtrate of the muscle which had been pulverized in a mortar after freezing in liquid oxygen. The results of these experiments are summarized in Tables III and IV.

TABLE IV

*Urinary Galactose and Galactose and Glucose in Gastrointestinal Tract of Rats after Administration of Sugars in 50 Per Cent Solution*

Time after sugar	Type of experiment	After galactose administration					After glucose administration		
		No of animals	Fed	Gastro-intestinal tract	Urine	Galactose retained	No. of animals	Gastro-intestinal tract	Urine
hrs.			mg.	mg.	mg.	mg.		mg.	mg
3	Sugar fed	3	1791	605	272	914	3	60 5	
	Control	5		7.9	3 5		3	5 2	
5	Sugar fed	6	1824	149	730	945	4	17.6	7 3
	Control	2		13.2	3.2				
7	Sugar fed	6	1872	93 0	863	919	4	8 3	4 2
	Control	2		8.8	3.7				
9	Sugar fed	2	1820	10.9	897	922	4	5 8	3 8
	Control	3			7.2				
24	Sugar fed	5	1785	7 8	825	952	4	3 3	12 2
	Control	1			15 5		4	2 8	

The liver glycogen is definitely higher throughout the 24 hour period in the glucose-fed animals although the discrepancy is least marked at the end of this interval. The muscle glycogen in both groups of the sugar-fed animals is considerably elevated over that of the controls. The rise occurs less rapidly in the galactose-fed rats though it may slightly exceed that of the glucose-fed ones at the end of 24 hours. We have no data as to whether this difference would become accentuated if the experiments had been continued longer after the carbohydrate administration. The muscle sugar is higher throughout with the galactose-fed animals—the maximum level being in the animals killed 5 hours after the sugar was given.

The galactose retained by the several groups is remarkably uniform. Approximately 50 per cent was excreted in the urine The

absorption required between 7 and 9 hours with the galactose-fed animals while it was practically finished after 5 hours with the glucose-fed ones. In the latter group, however, a much smaller amount of the sugar had been administered. No glucose was excreted in the urine after the administration of this sugar.

*Experiments on Rats Fed Diets High in Glucose, Galactose, and Lactose*—The main group of experiments was carried out on rats which were fed diets high in glucose, galactose, lactose, or glucose and galactose for a period of 7 to 12 days prior to the beginning of the fasting period. This is probably a more physiological procedure than the administration of a single large dose of a sugar. By its use it would enable one to determine more readily whether any difference in glycogenetic ability obtains between these sugars. The experiments were carried out at various times over a period of a year but the experiments in each of the five different series were done at the same time under uniform conditions usually on litter mates. Therefore, the experiments in each series are directly comparable with each other. Diet I, used for Series I and II, is as follows:

	per cent
Casein	20
Lard	20
Yeast	10
Cellu Flour <sup>1</sup>	2
Salt	4
Glucose, galactose, lactose, or glucose-galactose <sup>2</sup>	44

In the remainder of the experiments (Series III to V) the standard diet used for our stock animals was modified with the addition of the various sugars. As this already contained skim milk powder, some lactose was given in each group of experiments. Diet II was as follows:

	per cent
Whole yellow corn-meal	42
“ wheat flour	10
Powdered skim milk	20
Glucose, galactose, or lactose	20
Powdered alfalfa	4
Cod liver oil	2
Yeast (irradiated)	1
CaCO <sub>3</sub>	0 5
NaCl	0 5

<sup>1</sup> Omitted in Series II and replaced by sugar

<sup>2</sup> 22 per cent of each sugar

Table V gives the complete data of the individual experiments in one group of rats fasted 54 hours after the removal of the food. Because of lack of space only the average results are reported in the other tables (Tables VI to IX) which summarize the results of the experiments in which no fasting, 24, 48, or 72 hours of fasting occurred after the removal of the food prior to the killing. Because of the fact that the glycogenetic ability of galactose and lactose is a debatable question, we have considered it necessary to present

TABLE V  
*Liver and Muscle Glycogen in Rats Following a 54 Hour Fast (Series I)*

Experimental diet	Rat No	Body weight	Food intake per 100 gm rat		Glycogen	
			Per day	Last day	Liver	Muscle
		gm	gm	gm.	per cent	per cent
Glucose	210	150			0 18	0 19
	211	137			0 33	0 22
	212	131			0 18	0 17
Average . .			13 6	17 5	0 23	0 19
Galactose	222	143			0 32	0 33
	223	142			0 94	0 31
	224	121			1 06	0 33
Average .			13 0	15 7	0 77	0 32
Lactose	216	147			0 31	0 27
	217	138			0 29	0 30
	218	120			0 57	0 27
Average .			6 09	7 28	0 39	0 28

as completely as possible a summary on all of our data from which our conclusions are drawn.

The highest average glycogen content for both the liver and the muscle is almost invariably found in those animals which had previously been on the galactose diet. The poorest results were almost as uniformly obtained with the glucose-fed rats while those on the lactose or glucose-galactose régime usually occupied an intermediate position, as would be expected. In those animals which were killed without any preliminary fasting period, the

TABLE VI

*Summary of Experiments on Rats (Four in Each Case) without Any Fasting*

Series No. and sex	Experimental diet	Average weight	Average food intake per 100 gm rat per day	Glycogen		
				Liver	Muscle	Heart
		gm.	gm.	per cent	per cent	per cent
III♂	Glucose	181	3 71	4 03*	0 32	0 27
				3 54*		
	Galactose	167	3 78	2 69*	0 44	0 23
				2 89*		
V♂	Glucose-galactose	176	3 92	4 19*	0 43	0 24
				4 17*		
	Glucose	259	5 20	2 61	0 33	
	Galactose	238	4 96	2 63	0 40	
V♀	Lactose	255	4 63	4 12	0 31	0 25
	Glucose	165	5 48	3 60	0 35	
	Galactose	192	4 12	3 06	0 37	
	Lactose	178	4 89	3 00	0 36	0 23

\* Average of right and left portions of the liver in each group.

TABLE VII

*Summary of Experiments on Rats Fasted 24 Hours*

Series No and sex	Experimental diet	No of rats.	Average weight	Average food intake per 100 gm rat per day	Glycogen		
					Liver	Muscle	Heart
			gm.	gm	per cent	per cent	per cent
II♂	Glucose	3	211	4 82	0 13	0 19	0 59
	Galactose	3	183	7 72	0 37	0 29	0 52
	Galactose-glucose	3	195	9 57	2 12*	0 32	0 46
III♂†	Glucose	4	191	2 87	1 35†	0 29	0 33
					1 77†		
	Galactose	4	180	3 14	0 48†	0 43	0 33
					0 54†		
V♂	Galactose-glucose	4	170	4 16	0 41†	0 35	0 36
					0 44†		
	Glucose	4	221	5 16	0 22	0 23	0 30
	Galactose	4	216	5 56	0 65	0 30	0 39
V♀	Lactose	4	258	5 31	0 24	0 26	0 40
	Glucose	4	168	5 71	0 26	0 20	0 38
	Galactose	4	155	7 87	0 70	0 33	0 40
	Lactose	4	158		0 32	0 33	0 36

\* Abnormally high value, probably because of excessively high food intake on the day preceding fasting.

† Animals killed in afternoon.

‡ Average of right and left portions of the livers in each group.

liver glycogen was highest with the glucose-fed rats in two of the three groups of experiments. However, the muscle glycogen of the galactose-fed group was strikingly higher, being elevated as much as 40 per cent in one series of tests.

TABLE VIII  
*Summary of Experiments on Rats Fasted 48 Hours*

Series No and sex	Experimental diet	No of rats	Average weight	Average food intake per 100 gm rat per day	Glycogen		
					Liver	Muscle	Heart
			gm	gm	per cent	per cent	per cent
I ♂*	Glucose	3	140	11 5	0 23	0 19	
	Galactose	3	135	12 1	0 77	0 32	
	Lactose	3	135	6 34	0 39	0 28	
II ♂†	Glucose	3	215	5 43	0 13	0 20	0 56
	Galactose	3	191	6 49	0 12	0 23	0 46
	Galactose-glucose	3	200	4 32	0 13	0 21	0 58
IV ♂	Glucose	2	237	6 50	0 49	0 18	
	Galactose	4	200	5 60	0 89	0 30	
	Lactose	3	204	5 29	0 55	0 22	
IV ♀	Glucose	4	176	‡	0 12	0 22	
	Galactose	1	152	‡	0 38	0 25	
	Lactose	3	170	‡	0 22	0 26	
V ♀	Glucose	4	158	8 04	0 28	0 21	0 62
	Galactose	4	159	8 86	1 24	0 27	0 50
	Lactose	3	188	4 20	0 93	0 28	0 68

\* 54 hours fast.

† 45 hours fast.

‡ Food spilled.

In the majority of experiments in which 24 or 48 hours of fasting occurred prior to the killing of the rats, the liver glycogen is highest in the galactose-fed animals, intermediate in the lactose-fed ones, and least with the glucose-fed ones. In only one experiment (Series III, Table VII) was glucose superior, and this might be explained on the basis of a possible diurnal variation in glycogen, inasmuch as these animals were killed in the afternoon, while all of the others had been killed early in the morning. *In every*

case the average of the muscle glycogen is highest in the galactose- or lactose-fed group and the lowest results are invariably obtained with the animals which had been previously fed the high glucose diet. In a number of instances the mean of the muscle glycogen values is as great as 60 per cent more in the galactose-fed group and may exceed the glucose-fed controls by as much as 69 per cent (Series I, Table VIII).

TABLE IX  
*Summary of Experiments on Rats Fasted 72 Hours*

Series No and sex	Experimental diet	No of rats	Average weight	Average food intake per 100 gm. rat per day	Glycogen		
					Liver	Muscle	Heart
			gm	gm	per cent	per cent	per cent
III♂	Glucose	4	182	3 70	0 40*	0 23	0 51
					0 44*		
	Galactose	4	181	3 17	0 48*	0 28	0 52
.					0 49†		
	Lactose	4	173	5 31	0 62*	0 23	0 59
					0 53*		
V♂	Glucose	4	229	6 28	0 40	0 21	0 48
	Galactose	3	230†	5 42	0 36	0 24†	0 51
	Lactose	2	231	6 00	0 35†	0 27†	0 20
V♀	Glucose	3	185	6 58	0 32	0 26	0 45
	Galactose	4	161	8 18	0 30	0 25	0 60
	Lactose	4	179	5 69	0 70	0 28	0 63

\* Average of right and left portions of the liver in each group.

† Average of two animals only.

‡ Average of original four rats.

In two of the three series of experiments with 72 hours of fasting, the muscle glycogen is still highest in the galactose- or lactose-fed group, but the differences are small compared with the tests in which the periods of fasting were shorter. In two cases the liver glycogen is strikingly higher with the lactose-fed animals, although the galactose experiments differ little from the glucose ones.

The results on heart glycogen are so irregular that we have made no attempt to correlate them. In general, they show the well known response of the heart muscle, namely a consistent increase

in glycogen content as long as the fast progresses, concomitant with a decrease of this constituent in the skeletal muscle. No variation in the effect between these sugars could be noted.

*Experiments on Rats Fed on Galactose after Special Diets*—Three series of tests were made in which galactose was administered after varying periods of fasting following the ingestion of Diet I. Table

TABLE X

*Liver and Muscle Glycogen in Rats Killed 6 to 7 Hours after Galactose Administration Following a 48 Hour Fast*

Experimental diet	Rat No	Body weight	Food intake per 100 gm rat		Glycogen		Sugar retained
			Per day	Last day	Liver	Muscle	
		gm	gm	gm	per cent	per cent	per cent
I, glucose	207	188			1 05	0 25	60 1
	208	131			1 70	0 29	60 0
	209	120			1 70	0 30	62 5
Average			12 4	16 4	1 48	0 28	60 8
I, galactose	219	153			2 37	0 30	63 7
	220	141			2 67	0 33	65 0
	221	123			1 97	0 38	60 9
Average			11 8	15 0	2 34	0 34	63 2
I, lactose	213	154			1 46	0 32	70 9
	214	137			1 03	0 29	64 1
	215	130			1 29	0 36	69 4
Average			5 71	7 33	1 26	0 32	68.1

X gives the individual results on one series while the averages are summarized in Table XI.

The glycogen storage after the administration of galactose to the rats previously on the special diets employed in the previous section is in harmony with the results obtained in simple fasting. In every case the liver glycogen is definitely highest in the animals previously on the galactose régime. In both series where a galactose-glucose diet was employed, the liver glycogen occupies an intermediate position while the lone experiment on lactose shows

TABLE XI  
*Summary of Control Experiments on Fasting Male Rats and on Rats Similarly Treated but Fed 1.5 Mg of Galactose per Sq. Cm. of Body Surface 6 to 10 Hours before Being Killed*

Series No	Control experiments										Galactose feeding experiments									
	Experimental diet	No of rats	Average weight gm	Average food in- take per 100 gm rat per day	Glycogen			Time fasted before galactose admin- istration hrs	Time killed follow- ing galactose ad- ministration hrs	No of rats	Average weight gm	Food intake per 100 gm rat		Glycogen			Galactose retained			
					Liver	Muscle	Heart					Average per day gm	Average last day gm	Liver	Muscle	Heart				
I*	Glucose	3	140	13.6	0.23	0.19	48	6	3	140	12.4	16.4	1.48	0.28		60.8				
	Galactose	3	135	13.0	0.77	0.32			3	137	11.8	15.0	2.34	0.34		63.2				
	Lactose	3	135	6.09	0.39	0.28			3	141	5.71	7.33	1.26	0.32		68.1				
II	Glucose	3	201	4.82	0.13	0.19	24	9	2	224	6.03	6.98	1.53	0.27	0.64	52.6				
	Galactose	3	187	7.20	0.37	0.29			3	211	4.92	4.68	2.23	0.28	0.70	52.1				
	Galactose-glucose	3	191	9.30	2.12†	0.32	0.46			3	205	4.58	5.26	1.80	0.26	0.68	48.3			
II	Glucose	3	192	5.29	0.13	0.20	36	9	3	204	4.84	6.41	0.82	0.28	0.58	54.4				
	Galactose	3	194	6.09	0.12	0.23			3	201	6.32	7.95	1.65	0.27	0.48	59.5				
	Galactose-glucose	3	196	4.20	0.13	0.21			3	199	4.71	6.38	1.48	0.30	0.59	56.0				

\* Young rats. The large food intake is reflected in the rapid increase in weight during the experimental diet

† Abnormally high value, probably because of excessively high food intake on the day preceding fasting.



poorer results. This latter may be attributed to the fact that in some cases the animals refuse to take as much of the lactose food. In spite of this fact our other experiments with simple fasting have usually placed this sugar in a superior position to glucose. In this particular group of experiments on fairly rapidly growing rats, the lactose-fed animals failed to show an appreciable gain in weight during the time when the special diet was used while considerable increases took place in the glucose- and galactose-fed animals.

In Series I the muscle glycogen is also definitely the highest with the animals previously fed the galactose diet. The lactose-fed animals occupy an intermediate position. In the other two series of tests no differences in muscle glycogen could be noted although the liver glycogen had shown the characteristic variations. The rise in muscle glycogen after a single administration of galactose to the animals previously on the glucose diet is much more striking than it was with those rats which had been on the other foods. This suggests that a limiting factor may exist in the height to which the muscle glycogen may be raised. As the level in the control animals previously on the galactose or lactose diets approximates this value, additional galactose did not cause a further elevation. On the other hand since it was appreciably lower in the control glucose-fed rats, the ingestion of galactose was followed by a very considerable increase in this constituent.

*Effect of Long Continued Galactose Diet on Galactose Retention—* The question arises whether galactose would be retained in the rat to a larger extent if it was administered in the food over a prolonged period. If such a tolerance could be established, a single dose of this sugar should show a greater glycogen formation.

Litter mates after weaning (18 days old) were put on diets high in glucose, galactose, or lactose (Diet II). The galactose tolerance of each group was ascertained at the end of the 2nd and 4th months by feeding the animals 5 mg. of galactose per sq. cm. of body surface on a day when other food was withheld. The fast had begun at 5.00 p.m. on the previous night. The sugar was determined in the urine collected in metabolism cages during the 24 hour period subsequent to the sugar feeding. The results are reported in Table XII.

There is no evidence that the continued ingestion of a diet high

TABLE XII

*Galactose Retention in Rats Fasted 12 Hours after Long Continued Diets High in Glucose, Galactose, or Lactose*

Diet	Sex	Rat No	Litter No	Experiment I			Experiment II		
				Age	Body weight	Galactose retained	Age	Body weight	Galactose retained
				<i>days</i>	<i>gm</i>	<i>per cent</i>	<i>days</i>	<i>gm</i>	<i>per cent</i>
Glucose	M	486	2	70	152	45 7	131	235	27 6
	"	494	3	69	141	39 2	130	217	28 8
Average						42 4			28 2
Glucose	F	488	2	69	128	32 6	132	190	27 8
	"	492	3	68	120	41 0	131	166	31 1
	"	499	4	64	105	38 8	127	163	29 2
	"	502	4	64	76	39 4	127	162	34 9
	"	504	4	64	74	38 4	127	166	29 3
Average						38 0			30 5
Grand average						39 3			29 8
Galactose	M	481	1	71	104	30 9	132	178	26 8
	"	485	1	71	68	24 7	131	160	26 6
	"	489	2	70	172	30 3	131	246	29 5
	"	497	3	69	120	35 7	130	177	27 2
Average						30 4			27 5
Galactose	F	491	2	69	127	37 4	132	177	*
	"	498	4	64	74	31 1	127	150	39 5
	"	501	4	64	70	35 0	127	158	*
Average						34 5			39 5
Grand average						32 2			29 9
Lactose	M	484	1	71	90	37 8	132	197	36 0
	"	487	2	70	138	33 5	131	230	27 9
	"	505	4	64	69	46 3	126	169	45 3
Average						39 2			35 7
Lactose	F	490	2	69	117	31 9	132	175	31 7
	"	493	3	68	74	23 4	131	155	35 4
	"	500	4	64	77	52 3	127	154	27 9
Average						35 9			31 6
Grand average						37 5			33 7

\* Accidentally killed

in galactose increases the tolerance for this sugar. No sexual variability was noted in the ability to utilize galactose, although it would be interesting to compare its retention in the female during pregnancy and lactation. The group of animals was killed later after a 48 hour fast and the glycogen content of the liver and muscle in the various groups determined. These showed similar variations to the other series of animals. They comprise Series IV.

#### DISCUSSION

In the experiments reported on dogs previously fasted for 6 days, the administration of a single dose of galactose resulted in a more pronounced rise in the liver glycogen than was occasioned by that of an amount of glucose equal to the galactose retained. The maximum value, reached 12 hours after the galactose was administered, is 5.77 per cent, while the highest level in the glucose-fed control animals was 4.15 per cent. No consistent differences in muscle glycogen could be noted, perhaps because of our inability to obtain dogs sufficiently uniform in breed and age.

Although the experiments on rats, in which an amount of glucose equal to the galactose retained was given in a single dose, fail to show any superiority for the latter sugar as a glycogen former, much more convincing results were obtained after subjecting the animals to a diet containing a large percentage of glucose, galactose, or lactose (or glucose-galactose). Five series of experiments were conducted, in each of which three to eight rats were used with each sugar. Also in four of the series, groups of this number of animals were killed at several periods after the removal of food. In a large majority of the eighteen sets of tests, the animals previously on a galactose, lactose, or glucose-galactose diet showed a markedly higher level of liver glycogen than those previously on the glucose régime. If we exclude the three sets in which no fasting occurred and one in which no difference between the sugars could be noted, the liver glycogen was highest in the galactose- or lactose-fed group in all except two of the fourteen sets of experiments. One of these might be accounted for on a diurnal variation while the second one occurred in the 72 hour group (when the discrepancies between the different groups have become less marked). In almost every case both the galactose- and lactose-fed animals gave higher results—those with the latter sugar occupying an intermediate position.

However, the case for galactose becomes much stronger when one considers the muscle glycogen. In every instance in the eighteen experiments (including the ones in which no preliminary fasting period occurred) in which the lactose or galactose foods had been the previous dietary régime, the average muscle glycogen is higher than in the glucose-fed rats. In all fifteen experiments in which galactose was not administered after the special diets, the glucose-fed rats have the lowest level of muscle glycogen. This divergence in many cases is very great, showing results as much as 70 per cent higher with the galactose-fed animals. Such a discrepancy is even evident in some of the rats which were killed without any prior fasting. The values for muscle glycogen of the posterior muscles of the right rear leg of the rat are in the same range as those reported by Greisheimer (11) for the thigh muscles of non-fasted rats as well as those for the isolated leg muscles of dogs as reported by Major and Mann (12). They are lower than those of Cori (13) and Long (14) for the isolated gastrocnemius of rats. The divergence between our results and those of the latter investigator are somewhat less if his values are calculated as glycogen by the use of the factor, 0.927, as ours are done. On this basis his averages for 24 hour fasts are 0.487 per cent and for animals fasted 48 hours are 0.422 per cent. However, since Major and Mann (12) have demonstrated the fact that different muscles contain strikingly different amounts of glycogen, our values are not necessarily irreconcilable with those for the isolated gastrocnemius. Our muscle samples were taken in a similar manner in our various groups so that they are directly comparable with one another.

As far as we can note, there is nothing but the difference in the sugars composing the diet to account for the uniformly quantitative variations found in the glycogen. It is true that in some experiments the average food intake was higher in the galactose-fed animals. However, it should be remembered that a considerable portion of the sugar in these cases is lost in the urine. Although we have not made a study of the amount so excreted, it was observed that the galactose-fed animals drank more water and had a polyuria, both of which are indicative of a galactosuria. Moreover, rats never develop a tolerance for this sugar. Some of the experiments in which the intake of the galactose food is definitely lower than that of the glucose-fed animals show the most striking

differences in the level of glycogen. It is evident that difference in food consumption is not the cause of the variations in glycogen stores.

One might suggest that the galactose-fed animals were in poorer condition during the fast than the glucose-fed controls and so moved about less. As a consequence the glycogen store is more slowly depleted. Although this objection cannot be absolutely answered inasmuch as we did not use activity cages, the loss of weight should be a fair index of the extent of metabolism. In our animals, the weight loss was usually slightly greater with the galactose- and lactose-fed animals during the fast period than with the glucose-fed ones, which is inconsistent with a lower rate of metabolism in the former groups.

The discrepancies between our results and those of other investigators (2-4) may be ascribed partly to the longer periods which we have allowed to elapse after the ingestion of the sugar, partly by making the comparison of results when similar amounts are *retained* (in the dog experiments), rather than fed, but chiefly because of a more physiological procedure of incorporating these sugars in a diet and feeding them over a considerable period prior to the experiment rather than in administering them in a single unphysiological dose. Obviously, it is unfair to draw conclusions about the behavior of galactose in humans in whom only 4 per cent is excreted after doses of 37.5 gm. per sq. m. of body surface from experiments on rats in which more than 50 per cent is eliminated in the urine after 47 gm. per sq. m. of body surface (4.7 mg. per sq.cm.) are administered. In addition, it may be that galactose can only exert its most beneficial effect if it is metabolized with some other foodstuff, as the results of Corley seem to suggest (15). This is further supported by the recent results on humans of Harding and Grant (16) who showed that the moderate galactemia, which amounted to only 30 to 60 mg. per 100 cc. of blood after 50 gm. of galactose were taken orally, was actually lowered after 30 gm. of glucose were added to the dose of the former sugar. It was proved, moreover, that the total glycemia and the galactosuria were reduced.

If one may translate the results of the latter series into probable effect on humans, these experiments offer a satisfactory explanation for the more prolonged ketolytic effect of a single dose of galactose

in man (1). It is obvious that galactose could only exert its prolonged effect (which continued into the 3rd day after its administration) if it causes a quantitative or qualitative difference in the production of glycogen. One could hardly conceive that it would bring about this action by remaining for that length of time in the body as the free sugar.

#### SUMMARY

When glucose was administered to female dogs previously fasted for 6 days, the accumulation of liver glycogen was greater 6 hours after the ingestion of the sugar than it was in animals which retained a corresponding amount of galactose. However, the liver glycogen in the animals killed at intervals between 12 and 72 hours after the sugar feeding was higher in the dogs which had been fed galactose than it was in those which received glucose. Although the muscle glycogen seems to be definitely higher in the glucose-fed animals killed 6 and 12 hours after the administration of the carbohydrate, there are no consistent differences in the later periods. Experiments on rats following a single feeding of galactose failed to show any superiority of this sugar over glucose although the results obtained after 24 hours of fasting indicate that it is approximately as satisfactory.

In an extensive series of experiments on rats which had first been fed on diets high in galactose, lactose, or galactose-glucose for 7 to 12 days, the liver and muscle glycogen were almost invariably higher after periods of fasting up to 72 hours than they were in animals which had been on a similar diet with the sugar replaced by glucose. This is probably not to be traced to a difference in the amount of liver glycogen at the beginning of the fast as this was somewhat higher in two of three series of glucose-fed animals although the muscle glycogen was higher in the galactose-fed rats in these cases.

It would appear that the best explanation for these results is that the glycogen originating from galactose is *retained* longer than that formed from glucose, although in the experiments on dogs it appears to be more rapidly *synthesized* from the latter sugar. These results offer a satisfactory explanation for the superior ketolytic effect which has previously been noted for galactose in humans (1) as well as in fasting rats in which an appreciable ketosis

is artificially produced by the administration of diacetic acid orally or subcutaneously (17).

No increase in the amount of galactose retention was noted in rats placed on a high lactose or galactose diet at weaning and continued thereon for 3 months over that obtained with animals kept for the same period on a glucose diet.

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## **A DIFFERENTIAL LIPID ANALYSIS OF BLOOD PLASMA IN NORMAL YOUNG WOMEN BY MICRO- OXIDATIVE METHODS**

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The study of fat metabolism in human subjects has been largely confined to a consideration of the variations in blood lipids under different conditions. Most investigations have dealt with changes in a single lipid, cholesterol being probably the most intensively studied, but a few papers have appeared in which two or more lipids were simultaneously investigated in the same subject. Among these may be mentioned the work of Page, Pasternack, and Burt (20) in which total fatty acids, cholesterol, phosphatides, and iodine numbers were determined in eight subjects of both sexes. Okey and Boyden (18) studied variations in the total blood cholesterol, fatty acids, and phospholipid in sixteen women during menstruation. Man and Gildea (13, 14) have reported fatty acids and lipid phosphorus in normal men following ingestion of food. Other group lipid analyses include that of de Vass (23) on total and free cholesterol and phospholipids in blood serum of normal subjects and cancer patients, and that of Fahrig and Wachter (8) on lecithin, cholesterol, and neutral fat in human blood serum.

Such composite blood lipid analyses have considerable advantage over single lipid determinations in the interpretations of fat metabolism in relation to the blood. Recognizing this, the author has assembled, in the investigation to be reported below, a group of micromethods recently evolved by Bloor and his associates (3, 4, 6, 17, 24, 25), and from these devised a procedure whereby all the lipids known at present in plasma may be determined on a small amount of blood.



This procedure was then applied to an analysis of the lipids present in blood plasma of normal, young, non-menstruating, non-pregnant women. Unlike men, in whom the blood lipids remain fairly constant at definite levels, it has been found that in women there is a cyclic variation of the blood lipids in relation to menstruation. Okey and Boyden (18) demonstrated that during catamenia there is a fall in whole blood cholesterol with a tendency toward alterations in the fatty acids, while the phospholipids remain relatively constant. They conclude that, "the blood cholesterol level in women is to be considered as a variable rather than a constant." These findings have been confirmed by Kaufmann and Mühlbock (10) and later by Okey and Stewart (19). The present investigation was therefore undertaken to determine the extent of this variation in blood plasma lipids of young women under controlled conditions.

#### EXPERIMENTAL

*Subjects*—Eight young women varying in age from 20 to 38, with an average of 28, acted as subjects for the investigation. In each case it was ascertained that the previous menstrual period was 1 to 3 weeks past and that menstruation was of normal flow, duration, and occurrence, so that each subject was known to be within the 2 weeks between periods. A brief history was then taken and a physical examination made, including height, weight, temperature, pulse and respiration, blood count hematocrit, urinalysis, and blood Wassermann, to eliminate conditions which are known to affect the level of blood lipids (Bloor (5)), especially infection, cancer and tumor growths, anemia, thyroid dysfunction, uterine bleeding, etc. The subject thus selected as suitable for a study of normal plasma lipids was put on a normal balanced diet for a week and kept in bed for at least 24 hours before the test to offset the lipemic effect of exercise (Stewart, Gaddie, and Dunlop (22)) and emotion (Lyons (12)).

*Plasma Extracts*—Following a 16 hour fast, blood was withdrawn from the arm veins between 8.30 and 9.00 a.m., shaken in a flask with dry sodium citrate, and immediately centrifuged at 2000 revolutions for  $\frac{1}{2}$  to  $\frac{3}{4}$  of an hour. The plasma was drawn off and 10 cc. added slowly and with rotation to about 125 cc. of alcohol-ether, 3:1, both redistilled, after the method of Bloor

(3). The extract was filtered through an alcohol-extracted filter paper, the precipitate being washed several times with alcohol-ether, and the remaining solvent pressed out with a clean glass rod. After making up to 200 cc. volume, giving a 1:20 dilution of the original plasma, the extract was stored in clean bottles stoppered with alcohol-extracted corks. Iodine number estimations were made the same day the extract was prepared, but the lipids were determined later when convenient.

### *Methods*

A number of microlipid methods were considered for this study, based on different chemical principles of analysis such as colorimetry, nephelometry, acid titration, oxidation, and microscopic counts (Bloor (5)). In general there is a fairly consistent agreement in results obtained by the several procedures where one lipid is being estimated. However, where it is desired to calculate, from experimental figures, values for lipids not directly determined, there is sufficient variation between procedures to warrant serious consideration in the choice of methods. Whereas, at the present time, it is impossible to state by which method the correct lipid values are more closely approximated, there is an obvious advantage, both theoretical as well as practical, in a procedure composed of several methods, to have each of these based on the same principle. In place of having to consider a summation of errors inherent in each of the methods used, there is but one group of errors throughout. Likewise, having the determined values for each lipid in terms of the same unit facilitates calculation of lipids not directly estimated.

With this idea in mind, it was found that the oxidative procedure was the only one which could be applied to the determination of all the lipids known at present in blood plasma. Since it had previously been found that the microoxidative method for phospholipid was satisfactory (Boyd (6)), and a preliminary investigation demonstrated, as shown below, that a similar range of experimental error existed for the remaining methods, it was decided to adopt these methods for the present analyses. Total fatty acids, total cholesterol, free cholesterol, phospholipid, iodine number of total fatty acids, and iodine number of phospholipid fatty acids were determined directly. From these values were

calculated total lipid, neutral fat, cholesterol ester, neutral fat fatty acid, cholesterol ester fatty acid, and phospholipid fatty acid. It may thus be seen that a fairly comprehensive survey of plasma lipids was obtained by this procedure.

*Total Fatty Acids*—Total fatty acids and total cholesterol were jointly determined on a 20 cc. aliquot of the alcohol-ether extract by saponification with 0.1 cc. of saturated sodium hydroxide, acidification, and petroleum ether extraction after the method of Bloor (3). Six extractions, with 3 to 5 cc. of petroleum ether (b.p. 37–60°) each time, were found to remove all the oxidizable material. As these plasma extracts were quite clear, no appreciable difference in the result was obtained by allowing the petroleum ether extract to settle overnight and filtering, an added precaution of Kimmelstiel and Becker (11) for tissue analysis. From the petroleum ether extract the subsequent evaporation and oxidation were carried out as in the Bloor procedure. The reproducibility of the method in the hands of the author was determined on known alcohol-ether solutions of fatty acids and upon stock extract of blood plasma. It was found to give a mean recovery of 99 per cent on known solutions and a reproducibility varying within 2 per cent on the stock plasma extract.

*Total Cholesterol*—The oxidative method for total cholesterol by precipitation of cholesterol as the digitonide (Okey (17)) was found to offer the most difficulty in the way of reproducibility and accuracy of results, but a procedure was finally developed which proved quite satisfactory. A number of modifications were adopted from the method of Yasuda (24) and from personal communications with Professor W. R. Bloor, Mr. L. C. Miller, and Mr. P. L. McLachlan of the Department of Biochemistry, as well as changes introduced by the author. The procedure as finally used was as follows: A 10 cc. aliquot of the alcohol-ether extract was saponified in Erlenmeyer flasks on the steam bath with 0.1 cc. of saturated sodium hydroxide. Freshly prepared sodium ethylate may also be used but this reagent on standing becomes unsatisfactory for the saponification (16). When the volume of the mixture reached 3 to 4 cc., usually in about  $\frac{1}{2}$  hour which, as shown by Yasuda (24), is sufficient for complete saponification, the mixture was acidified with 1 cc. of 1:3 sulfuric acid. The flask was then placed to one side on the steam bath out of contact

with active steam and heated at this relatively lower temperature until the volume reached 1 cc., after which it was completely extracted with petroleum ether as in the fatty acid procedure above, a drop of phenol red being added for convenience in separating the petroleum ether layer (15). To the combined petroleum ether extracts in a clean 125 cc. Erlenmeyer flask were added 5 cc. of a 0.2 per cent solution of Merck's digitonin (16) in 50 per cent alcohol and the mixture evaporated almost to dryness on the steam bath. A gentle stream of air proved of value in the terminal stages of this evaporation. 10 cc. of distilled water were then added, caution being observed to prevent the water running down the sides of the flask over which the flakes of precipitate would otherwise be distributed, and the contents brought to a gentle boil for 2 minutes with continuous rotation of the flask. In this manner excess digitonin was dissolved and the precipitate reduced to a fine granular suspension. Occasionally it was necessary to dislodge, with a clean glass rod, portions of the precipitate which had remained adherent to the bottom or sides of the flask.

After allowing the mixture to cool, 20 cc. of redistilled acetone were added and the flask rotated vigorously, resulting in a certain amount of agglutination of the suspension which was then filtered with suction through a sintered glass filter of the type "4G4, Schott and Gen., Jena" and washed twice with acetone and ether, as in the Yasuda (24) procedure. The rate of filtration was limited to roughly 125 drops per minute, since it has been found that a faster rate produces lower values (15). The filter was then placed within a copper coil steam-jacket, redistilled methyl alcohol added, and steam passed through the coil until the solvent began to boil, when it was drawn into a 125 cc. glass-stoppered Erlenmeyer oxidation flask through a glass suction head inserted between the filter and the flask to facilitate connections with the water vacuum pump. This arrangement was essentially similar to that used by Yasuda, but methyl alcohol has proved to be a more convenient solvent for the cholesterol digitonide than absolute alcohol (16). After washing the filter twice with methyl alcohol, the filtrate and washings were evaporated to dryness, traces of methyl alcohol removed with a current of air, and the lipid oxidized as above. Yasuda's observation that a half hour's heating in the electric oven is necessary for complete oxidation was

confirmed, less heating resulting in lower recoveries on known amounts of cholesterol. With the procedure as above outlined, it was found that 96 per cent of a known amount of cholesterol palmitate added to a plasma extract could be recovered, while determinations on a stock alcohol-ether extract of blood plasma gave figures which could be duplicated within 2 per cent.

*Phospholipid*—Phospholipid was determined on 20 cc. aliquots of the plasma alcohol-ether extract by Bloor's method (4) with the modifications as introduced by Boyd (6). The acetone mother liquor and acetone washings after precipitation of the phospholipids were quantitatively transferred to a 125 cc. Erlenmeyer flask and this fraction used for estimation of free cholesterol as described below. The experimental errors of this method have been previously reported by the author (6).

*Free Cholesterol*—Free cholesterol was estimated on 20 cc. aliquots of the alcohol-ether extract after removal of the phospholipids as described above, 5 cc. of 0.2 per cent digitonin added, and the procedure carried out as from this stage for the total cholesterol. Values obtained by this procedure checked within the experimental error with values derived when free cholesterol was directly precipitated from the alcohol-ether extract. With the acetone washings, however, time is saved in digitonin precipitation and the digitonide precipitate requires less washing, since phospholipid impurities are not present. By this procedure 96 per cent of known amounts of cholesterol could be recovered, while duplicate determinations on stock plasma extract checked within 4.5 per cent.

*Iodine Numbers of Total Fatty Acids*—Iodine numbers were determined by the method of Yasuda (25) with 50 cc. of alcohol-ether extract, the estimations being made on the same day the extract was prepared. It was found by applying the method to known amounts of cholesterol that the theoretical iodine number of cholesterol was approximated within 7.5 per cent on repeated estimations when a sufficient amount of lipid was used to produce a difference of 1 cc. or more in the final titration with 0.02 N sodium thiosulfate, while titration differences of less than 0.5 cc. were found unreliable. Duplicate determinations agreed within 3.3 per cent. Calculation of the iodine number of plasma fatty acids was based on Yasuda's formula after computing and sub-

tracting from the titration difference the cc. of 0.02 N sodium thiosulfate due to the iodine number (theoretical) of total cholesterol in the aliquot (factor, 1 mg. of cholesterol equivalent to 0.26 cc. of 0.02 N sodium thiosulfate).

*Iodine Numbers of Phospholipid Fatty Acids*—Phospholipids were precipitated and dissolved in moist ether from a 50 cc. aliquot of plasma extract as in the procedure by Boyd (6). 5 cc. of redistilled alcohol were added to the moist ether solution of the phospholipids in a small Erlenmeyer flask and the lipid saponified with 0.1 cc. of saturated sodium hydroxide. Direct determinations of iodine number on whole phospholipid, as described by Yasuda (25), were found to give erratic and variable results. After saponification the phospholipid fatty acids were extracted with petroleum ether, evaporated, redissolved in 1 cc. of chloroform, and the iodine number determined with the Rosenmund-Kuhnhehn reagent as used by Yasuda. On a number of extracts the phospholipid fatty acids were directly determined, but it was found that similar values for the iodine number could be obtained by computing the phospholipid fatty acids as two-thirds of the phospholipid. Duplicate determinations on stock alcohol-ether extract were found to agree within 5 per cent.

*Calculated Lipids*—From the experimental values obtained by the above procedures the further distribution of the plasma lipids was found by calculation. Combined cholesterol (cholesterol of cholesterol ester) levels were obtained by subtracting the value for free cholesterol from that for total cholesterol and the figure for cholesterol ester fatty acids may be computed as 0.67 times combined cholesterol (the cholesterol ester fatty acids being calculated as oleic and  $C_{18}$  acids). The sum of the phospholipid fatty acids as above calculated plus the cholesterol ester fatty acids subtracted from the total fatty acids gives the value of the neutral fat fatty acids which constitute, on the average, 95 per cent of neutral fat, thus giving a figure from which neutral fat may be calculated. The total lipid may now be determined as the sum of the phospholipid plus the neutral fat plus the total cholesterol plus the cholesterol ester fatty acids.

### Results

In Table I are recorded the results of a series of analyses of blood plasma on eight normal young women by the methods as de-

TABLE I

*Composition of Blood Plasma Lipids in Normal Young Women*

The results are measured in mg per 100 cc of blood plasma.

Subject	Total lipid	Composition of total lipid										Iodine No	
		Neutral fat	Fatty acids					Cholesterol			Phospho-lipid		
			Total	Phospho-lipid	Cholesterol ester	Neutral fat	Total	Com-bined	Free				
D. E.	730	229 3	457	157	82 8	217 2	182	123 5	58 5	236		85 7	105 6
C. B.	546	159 7	335	119 6	63 8	151 6	143	95 2	47 8	179 6		78 3	117
J F.	521	155 4	332	134 3	50 0	147 7	113 6	74 6	39 0	202		64 5	117
M. H.	645	128 6	370	151 4	96 5	122 1	192	144 2	47 8	228		102 2	98 5
E. J.	422	78 3	245	120 5	50 2	74 3	112	74 8	37 2	181		91 8	167 2
M. F.	590	146 8	347	121	86 4	139 6	175	129	46 0	181 6		77 4	150 7
I. M.	614	136	347	122 4	96 2	128 4	195	143 6	51 4	187		96 2	106 7
E. A.	642	196	391	113 3	91 2	186	184 6	135 9	48 7	170		112	131 6
Mean	589	153 7	353	129 9	77 1	145 9	162	115 1	47 1	195 7		88 5	124 2
Standard deviation	87 2	42 2	55 8	15 1	18 3	39 8	32 1	27 4	6 28	22 5		40 2	42 7
Standard deviation, per cent	14 8	27 3	15 7	11 6	23 7	27 3	19 8	23 8	13 4	11 5		45 3	18 3

scribed above. For each lipid the mean value has been determined and from this the standard deviation calculated by the formula  $\vartheta = \sqrt{(\Sigma (x)^2)/n}$ , where  $x$  represents the variation of each value from the mean,  $n$  the total number of readings,  $\vartheta$  the standard deviation, and  $\Sigma$  a summation symbol (7). For comparative purposes the per cent of the standard deviation over the mean has also been given. Since the variation of each method has been determined above, single estimations only need be given for the plasma lipids.

The total lipid content of the plasma varied from 0.4 to 0.7 per cent with a mean value of 589 mg. per cent and with  $\vartheta$  (standard deviation) 87.2 or 14.8 per cent. The total lipid was composed as follows:

	<i>per cent</i>
Phospholipid	33 2
Cholesterol ester	32 7
Neutral fat	26 1
Free cholesterol	8 0

Total fatty acids of plasma averaged 353 mg. per cent, varying from 245 to 457 mg. per cent, with a standard deviation of 55.8 or 15.7 per cent of the mean. In 1921, Bloor (1) reported blood plasma total fatty acids in normal men as 380 mg. per cent, while Man and Gildea (14) found, in a review of the results by various authors, an average value of 330 mg. per cent by nephelometric methods. Using a modified Stoddard and Drury acid titration technique (13), the latter investigators determined total fatty acids in human blood serum as 11.9 milli-equivalents or 320 mg. per cent. With the Bang-Bloor oxidative method, Page, Pasternack, and Burt (20) found the serum fatty acids of normal fasting men and women ranged between 243 and 470 mg. per cent. From the present investigation it was found the total fatty acids were distributed as follows:

	<i>per cent</i>
Neutral fat fatty acids	41 4
Phospholipid " "	36 8
Cholesterol ester fatty acids	21 8

The glycerides constituted approximately one-quarter of the total lipid with a mean value of 153.7 mg. per cent and a standard deviation of 42.2, varying from 78.3 to 229.3 mg. per cent. Total



cholesterol showed less variation, having an average value of 162 mg. per cent with extremes of 112 and 195 mg. per cent. Bloor (1) reported 220 mg. per cent for total cholesterol in normal men and Page *et al.* (20) found the level varied between 137 and 245, both investigators using the Bloor-Liebermann-Burchard colorimetric method (3). The digitonin method, as employed in the present investigation, has been shown to give slightly lower values for cholesterol than the above colorimetric reaction (16). The total cholesterol was composed of 29.3 per cent free cholesterol which gave a mean value of 47.1 mg. per cent and varied between 37.2 and 58.5 mg. per cent. There was little tendency to variation in the level of this lipid, the standard deviation, 6.28, being 13.4 per cent of the mean. Phospholipid exhibited the least tendency of all the lipids to variation: the mean value obtained was 195.7 mg. per cent, the extremes 170 and 236 mg. per cent, and the standard deviation, 22.5, constituted 11.5 per cent of the mean. By nephelometric methods, Bloor (1) reported a value of 220 mg. per cent for normal men, while Page *et al.* (20) found a range of 238 to 309 mg. per cent; by estimating the lipid phosphorus Man and Gildea (14) calculated the phospholipid fatty acid from which it may be shown that they obtained a mean value of 247 mg. per cent for serum phospholipid. Values for serum lipids are, in general, somewhat higher than those for plasma (13, 14).

In contrast to the relative constancy of the lipid values noted above, the iodine numbers of plasma fatty acids showed a more marked variation. The iodine numbers of the total fatty acids varied from 112 to 64.5 with an average of 88.5, a standard deviation of 40.2, and a 45.3 per cent standard deviation. On the other hand, the iodine numbers of the phospholipid fatty acids showed less variation, having an average value of 124.2, extremes of 98.5 and 167.2, a standard deviation of 22.7, and 18.3 per cent *σ*. Page *et al.* (20) found the iodine numbers of serum total fatty acids varied between 98 and 132, while the iodine numbers of phospholipid fatty acids ranged from 52 to 87.

#### DISCUSSION

A certain amount of evidence has been presented in recent years to indicate that under controlled conditions the blood lipids are maintained relatively constant at definite levels. In 1923, Bloor

(2) found that there was little tendency to variation in the distribution of plasma fatty acids in cows, sheep, pigs, and dogs, this investigation confirming earlier work. Glusker (9) was able to show that in dogs kept on a standard diet at constant weight, the total fatty acid varied by a 13 per cent standard deviation and total cholesterol, under the same conditions, varied within a 28 per cent standard deviation. Somewhat later on the same group of dogs it was demonstrated by Boyd (6) that the phospholipid level was also maintained constant within a 17.5 per cent standard deviation. Earlier in this paper it has been shown that in the human female, under controlled conditions, a similar or even greater degree of constancy exists among the plasma lipids. In women it has been shown that total fatty acids varied by 15.7 per cent  $\sigma$ , total cholesterol by 19.8 per cent  $\sigma$ , and phospholipid by 11.5 per cent  $\sigma$ . In addition to the lipids mentioned above, the present report includes means and variations for the remaining known lipids of blood plasma together with similar figures for iodine numbers. Of all the lipids, neutral fat and cholesterol ester were found to be the most variable.

In the rat, Sinclair (21) found that the iodine number of the tissue phospholipid fatty acids was markedly affected by food fats and correspondingly it has been shown above that the iodine number of the phospholipid fatty acids tends to be more constant. If diet acts on plasma lipids as demonstrated on the tissues by Sinclair, one would expect, under controlled diet for 1 week, a constancy in the iodine number of the phospholipid fatty acids, a condition corroborated experimentally above. In 75 per cent of cases a phospholipid value lower than the mean was associated with an iodine number for phospholipid fatty acids higher than the mean or *vice versa*, suggesting that the iodine number of the phospholipid fatty acid varies inversely as the amount of phospholipid.

As stated above, Bloor (2) and others have emphasized that there tends to be a constant relation between the various constituent lipids of plasma and tissues. In Table II a series of ratios between the several lipids studied has been computed and for each ratio the mean value with the standard deviation and per cent  $\sigma$  determined. For the purpose of ascertaining the constancy of a ratio, it may be concluded that where the per cent standard devia-

tion of the ratio is less than the per cent  $\delta$  for each of the lipids in the ratio, then the ratio is more constant than each of its constituent lipids. Thus the ratio, total lipid to total fatty acid, with a mean value of 1.67, has a 4.0 per cent  $\delta$ , while the total lipid has 14.8 per cent  $\delta$  and total fatty acids 15.7 per cent  $\delta$ , indicating that the ratio tends to be constant. By application of the same criterion, it has been found that the ratios, total fatty acid to cholesterol and combined cholesterol to total cholesterol, also exhibit a degree of constancy, while the ratios, total fatty

TABLE II  
*Ratios between Blood Plasma Lipids in Normal Young Women*

Subject	Total lipid to total fatty acid	Total fatty acid to neutral fat	Total fatty acid to phospholipid	Total fatty acid to cholesterol	Phospholipid to cholesterol	Combined cholesterol to total cholesterol
D. E.	1 60	2 00	1 97	7 80	4 03	0 68
C. B.	1 63	2 10	1 87	7 00	3 75	0 67
J. F.	1 57	2 14	1 64	8 52	5 18	0 66
M. H.	1 74	2 88	1 62	7 73	4 78	0 75
E. J.	1 72	3 13	1 35	6 58	4 87	0 67
M. F.	1 70	2 36	1 91	7 53	3 95	0 74
I. M.	1 77	2 55	1 86	6 75	3 64	0 74
E. A.	1 64	2 00	2 30	8 03	3 49	0 75
Mean	1 67	2 39	1 81	7 49	4 21	0 707
Standard deviation	0 067	0 40	0 26	0 63	0 60	0 039
Standard deviation, per cent	4 0	16 7	14 4	8 5	14 3	5 5

acid to neutral fat and total fatty acid to phospholipid, were about as variable as the lipids themselves. The ratio, phospholipid to cholesterol, was more variable than its constituent lipids. It may therefore be concluded that under the controlled conditions of these experiments the variations in the lipid ratios were identical with the variations in the lipids themselves, and that the ratios are significant for comparative purposes only where one or other lipid level is altered by changed conditions in the experiment. It is obvious, also, that little value can be attached to the so called constancy of the ratio unless a critical standard, as described

above, be applied, and the value of such ratios, as they appeared in the literature, may thus be seriously questioned.

## SUMMARY

A procedure has been described for the complete lipid analysis of small amounts of blood plasma based on oxidative methods and the advantage of using similar methods in such an analysis discussed. It was found necessary to modify a number of the published methods, particularly the digitonin method for total cholesterol which has been described in detail. The experimental errors and reliability of each procedure have been reported.

By application of this differential lipid analysis to blood plasma of eight normal young women under controlled conditions of exercise, rest, and diet, the following mean values were obtained:

Total lipid, <i>mg. per cent</i>	589
Neutral fat, " " "	154
Total fatty acid, <i>mg. per cent</i>	353
Phospholipid fatty acid, <i>mg. per cent</i>	130
Cholesterol ester fatty acid, <i>mg. per cent</i>	77
Neutral fat fatty acid, <i>mg. per cent</i>	146
Total cholesterol, <i>mg. per cent</i>	162
Combined cholesterol, <i>mg. per cent</i>	115
Free cholesterol, <i>mg. per cent</i>	47
Phospholipid, <i>mg. per cent</i>	196
Iodine No of total fatty acids	88 5
" " " phospholipid fatty acids	124

By computing the standard deviations it has been shown that the plasma lipids and ratios between the plasma lipids are maintained constant, within certain limits, under the controlled conditions of the experiment. The values obtained have been compared with published figures and a discussion relative to the function of the various lipids included.

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# **SUBSTITUTED HYDRAZINE DERIVATIVES OF THE HEXURONIC ACIDS\***

## **PHENYLHYDRAZINE AND *p*-BROMOPHENYLHYDRAZINE DERIVATIVES OF *d*-GALACTURONIC ACID AND *p*-BROMOPHENYLHYDRAZINE DERIVATIVES OF *d*-MANNURONIC ACID**

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### **INTRODUCTION**

Progress in the chemistry and biochemistry of the hexuronic acids has been inhibited in part because well defined derivatives are lacking or difficult to prepare. The characterization and identification of the uronic acids (especially from reaction mixtures in which they may be present in small amounts) would be placed on a firmer basis if more suitable derivatives were available. This need is especially illustrated by the difficulties usually encountered in the identification of the aldehyde sugar acid components of the complex polyuronide substances, since an inevitable destruction of the free uronic acids liberated accompanies their hydrolysis (1).<sup>1</sup>

The limitations of the methods available at present can be illustrated by a review of the most important papers involving the isolation and identification of a uronic acid. Only some of the more salient points need be mentioned.

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<sup>1</sup> This paper gives references to the observations of various investigators on this question. It is important to note here that Dr. P. A. Levene deals at some length on the difficulties involved in establishing the presence of *d*-glucuronic acid in chondrosin (2). The difficulties referred to by Levene in his studies on the constitution of the mucoproteins parallel in a general way our observations and those of others on the polyuronide substances.

In the researches on the aldobionic acids obtained from the soluble specific polysaccharide substances of certain microorganisms (3, 4) the presence of the uronic acid was usually not established by direct isolation. In most cases it was necessary to oxidize the sugar acid component to the corresponding dicarboxylic acid, which was in turn isolated and characterized. The same method has frequently been employed to identify the uronic acid present in various plant gums and mucilages (5-10) and the alginic acids of marine algæ (11, 12). The limitations of the oxidation procedure were pointed out by Butler and Cretcher (7) in their study on the composition of commercial cherry gum. The quantitative analysis of the cherry gum acid showed a uronic acid content of 30.0 per cent, yet they were unable to establish the nature of the uronic acid present.

Ehrlich (13) in an extensive review article on the biochemistry of galacturonic acid and the pectin substances, has pointed out that in certain cases the reported occurrence of uronic acids in hemicelluloses and other cell wall substances must be questioned. This dubiety is justified since the characterizations were frequently inferred from color reactions and the formation of derivatives that were not well defined.

It is significant to note that the supposed occurrence of *d*-glucuronic acid in the alginic acid of various species of brown algæ is still an open question, due to variations in the physical constants exhibited by some of the alkaloidal salts of *d*-glucuronic and *d*-mannuronic acid in the hands of different investigators (12, 14-17).

The selection of the derivative to be used for the purposes of isolating the uronic acid is determined in part by the chemical nature of the accompanying substances. Thus in the presence of other unrelated acidic substances the use of a derivative involving combination with the carboxyl group would not lead to an easy isolation of the hexuronic acid.

The hexuronic acids have three functional groups, the carboxyl, the alcoholic hydroxyls, and the aldehydic or ketonic carbonyl. Reactions directed to combination with the carboxyl group resulting in salt formation have found considerable application. Thus in the preparation of *d*-glucuronic, *d*-galacturonic, and *d*-mannuronic acid from natural sources with a high uronic acid content, the formation of inorganic salts, particularly the barium and cal-

cium salts, has proved to be quite successful (15, 16, 18-23). This procedure, however, cannot usually be employed for isolation and characterization purposes when small quantities are involved since the barium and calcium salts have so far not been obtained in a distinctly crystalline condition.

The characterization of the uronic acids through the use of alkaloidal salts has been successful in some investigations. On the other hand, confusion has arisen in certain instances, particularly in the case of *d*-mannuronic acid (12, 14-17).<sup>2</sup>

The identification of *d*-mannuronic acid as a constituent of various marine algæ has been attempted in the majority of cases, through the use of alkaloidal salts (11, 12, 14, 17). Nelson and Cretcher (14, 24) have reported the isolation of *d*-mannuronolactone from the alginic acid of several species of marine algæ. Schoeffel and Link have obtained the  $\alpha$  and  $\beta$  forms of the free acid from the alginic acid of *Macrocystis pyrifera* and *Fucus serratus* (15). The validity of the values assigned to the physical constants of *d*-mannuronolactone and its brucine and cinchonine salt by Nelson and Cretcher (14) has been corroborated by the synthesis of *d*-mannuronolactone from *d*-mannose by Niemann and Link (16). In all cases the physical constants of the lactone, the brucine and cinchonine salts of the naturally occurring acid, were in agreement with the constants exhibited by the same compounds prepared from the acid obtained by synthesis.

The variation in the melting points and rotations of the alkaloidal salts of *d*-mannuronic acid reported by various investigators (11, 12, 14, 16, 17) may be due to several factors. As has been previously pointed out, the method of drying greatly influences the melting point of the cinchonine and brucine salts (16). Further, as Nelson and Cretcher have suggested, when polymers of *d*-mannuronic acid are present alkaloidal salts with higher melting points are obtained (14).

As mentioned above, it has been noted frequently (14, 15, 23, 24) that the barium salt of *d*-mannuronic acid obtained by the hydrolysis of the complex polymannuronides (the alginic acids) are invariably contaminated with the barium salts of lower polymers. When the alkaloids are used to characterize *d*-mannuronic

<sup>2</sup> Specific reference to the cases in point are given in these papers.



acid, the presence of salts of the lower polymers cannot be revealed with certainty by elementary analysis. On the other hand, if the lower polymers represent complexes wherein the *d*-mannuronic acid units are linked in part through the aldehyde group, the use of *p*-bromophenylhydrazine would yield discriminating derivatives. The presence of a polymer or polymers would be detected by an elementary analysis of the hydrazine compound. In general, reactions directed toward combination with the *carboxyl group alone* do not permit ready and exact characterization of a hexuronic acid.

Reactions with the alcoholic hydroxyl groups are restricted to relatively pure compounds in the sugar acid group. While these are of the highest importance in constitutional studies (25-27) they are at present of limited value for direct characterization and identification purposes.

The property of the aldehydic or ketonic carbonyl of the sugar group to form hydrazones or under certain conditions osazones, as illustrated by the classical studies of Emil Fischer (28), has in the past been extensively and fairly successfully applied to one naturally occurring member of the hexuronic acids, namely *d*-glucuronic acid (29-32). In contrast to the numerous substituted hydrazine derivatives of *d*-glucuronic acid the literature lists only two corresponding compounds of *d*-galacturonic acid (29, 30). Ohle and Berend (33) described the phenylhydrazine salt of the phenylosazone of *d*-galacturonic acid and indicated the existence of the phenylhydrazine salt of the phenylhydrazone.<sup>3</sup> In a recent article Neuberg and Collatz (35) mentioned the 2,4-dinitrophenylhydrazone of *d*-galacturonic acid but did not report their experimental procedure.

In their first note on the constitution of vitamin C (originally called hexuronic acid, now ascorbic acid (36)) Hirst and Reynolds (37) stated that a *p*-bromophenylhydrazine derivative of *d*-galacturonic acid, comparable to the *p*-bromophenylosazone barium salt of *d*-glucuronic acid was formed, by following the procedure

<sup>3</sup> Ohle and Berend (33) were the first to record that *d*-galacturonic acid yields a colored lead salt with basic lead acetate. Ehrlich (34) has used this color reaction as the basis for a qualitative test for *d*-galacturonic acid. Ehrlich apparently was not aware of Ohle and Berend's observation, since he did not cite their work.

of Goldschmiedt and Zerner (38). The compound isolated was not described in detail. In the light of the experimental results presented in this paper we are of the opinion that Hirst and Reynolds most likely did not have the *p*-bromophenylosazone barium salt in hand, but that the "yellow powder" mentioned is probably the highly insoluble barium *p*-bromophenylhydrazone *d*-galacturonate.

Bird and Haas (12) failed to prepare the *p*-bromophenylhydrazone of *d*-mannuronic acid and to the best of the authors' knowledge, no other attempts have been reported.

In the extensive program on the chemistry and biochemistry of the free and combined uronic acids under way in this laboratory, we have included a study of the substituted hydrazine derivatives. The investigations have been directed toward the objective of finding derivatives of the various naturally occurring hexuronic acids that can be prepared with ease and that also possess desirable physical and chemical properties. The first incentive to explore the field of uronic acid derivatives more thoroughly, arose through the observations of one of us (K.P.L.) that the corn seedling (*Zea mays*) contains small quantities of uronic acids in the cell wall, combined in the pectin fraction and possibly also to other polysaccharide substances. In addition some uncombined uronic acid (possibly *d*-glucuronic acid) occurs in the cell sap (39). These observations were originally put forward with reserve, since it was not possible to establish definitely, with the derivatives of the uronic acids available at the time, which uronic acid was involved.

In this communication we describe the preparation and properties of the following derivatives: Section I, (a) barium phenylhydrazone *d*-galacturonate, (b) phenylhydrazine phenylhydrazone *d*-galacturonate, (c) *d*-galacturonic acid phenylhydrazone, (d) phenylhydrazine phenylosazone *d*-galacturonate, (e) barium phenylosazone *d*-galacturonate; Section II, (a) barium *p*-bromophenylhydrazone *d*-galacturonate, (b) *p*-bromophenylhydrazine *p*-bromophenylhydrazone *d*-galacturonate, (c) *p*-bromophenylhydrazone *d*-galacturonic acid, (d) *p*-bromophenylhydrazide *p*-bromophenylhydrazone *d*-galacturonic acid; Section III, (a) barium *p*-bromophenylhydrazone *d*-mannuronate, (b) *p*-bromophenylhydrazone *d*-mannuronolactone, (c) *p*-bromophenylhydrazine *p*-bromophenylhydrazone *d*-mannuronate, and (d) *p*-bromophenylhydrazide *p*-bromophenylhydrazone *d*-mannuronic acid.

In the light of the results embodied in this communication it is interesting to note that Ehrlich, the first investigator to obtain *d*-galacturonic acid in a crystalline condition, stated in his original publication (40) that in contrast to *d*-glucuronic acid, no difficultly soluble (or relatively insoluble) crystalline derivatives of *d*-galacturonic acid could be obtained with phenylhydrazine or *p*-bromophenylhydrazine. As far as we are able to ascertain from Ehrlich's numerous later publications, he has not explored further the preparation of substituted hydrazine derivatives of *d*-galacturonic acid.<sup>4</sup> This investigation is being continued and the preparation and properties of the alkaloidal salts of several substituted phenylhydrazones will be reported in a future communication.

#### EXPERIMENTAL

The procedures used below are simple adaptations of those originally described by Fischer (28) and later recompiled by van der Haar (42). In all experiments freshly distilled phenylhydrazine was used. The *p*-bromophenylhydrazine, and the phenylhydrazine hydrochlorides were also recrystallized immediately before they were used. These precautions are *indispensable*. It is possible that the unsuccessful attempts of other investigators in this field have been due to the failure to observe these conditions.

The preparations of *p*-bromophenylhydrazine (either the free base or the hydrochloride) offered by the Eastman Kodak Company, or Akatos, Inc., New York (agents for Kahlbaum), are invariably too impure to be used directly. However, we have experienced no difficulty in purifying the preparations marketed by these companies.

The barium *d*-mannuronate and the *d*-mannuronolactone used in this study were prepared after the procedure of Schoeffel and Link (23). The *d*-galacturonic acid and the barium salt were prepared from a polygalacturonide preparation obtained from citrus pectin by using the rapid practical procedure of Link and Nedden (19).<sup>5</sup>

<sup>4</sup> Ehrlich's most recent review article wherein reference is made to all of his publications is in the book by Klein (41).

<sup>5</sup> Ehrlich and Guttman (43) commented adversely on our method for the preparation of *d*-galacturonic acid (19, 20). They are apparently unaware of the fact that the commercially available citrus pectin preparation which they have recommended as the starting material is identical

All analyses were conducted through the use of the Pregl micro-methods. The individual preparations were dried for 5 hours at 60° on the Pregl block prior to the analysis. Rotations were determined with a Franz Schmidt and Haensch (Berlin) quartz wedge saccharimeter equipped with the Ventzke scale. The new electric sodium lamp made by the same firm was used as the source of light. The melting points were made with the Thiele apparatus with an inclosed scale thermometer. In every case the temperature was raised at the rate of 6° per minute.

### *I. Phenylhydrazine Derivatives of d-Galacturonic Acid*

#### *(a) Preparation of Barium Phenylhydrazone d-Galacturonate.*

*Procedure 1*—0.58 gm. of phenylhydrazine was dissolved in a mixture of 12 cc. of water and 3.5 cc. of 50 per cent acetic acid. A solution of 1.45 gm. of barium *d*-galacturonate and 6.0 gm. of barium acetate in 15 cc. of water was then added to the hydrazine reagent. Within 10 minutes a heavy granular precipitate was formed which remained unchanged on the addition of 30 cc. of 95 per cent ethyl alcohol. After standing for 3 to 4 hours the precipitate was filtered off, washed successively with water, absolute ethyl alcohol, and ether. The product was finally dried over  $P_2O_5$  under 15 mm. pressure at 60°. 0.90 gm. of the derivative was obtained, whereas a theoretical yield requires 1.95 gm.

*Procedure 2*—0.50 gm. of phenylhydrazine phenylhydrazone *d*-galacturonate ((Section I, *b*) see below) was dissolved in 25 cc. of 60 per cent ethyl alcohol and the resulting solution titrated to a phenolphthalein end-point with 0.2 *N* barium hydroxide. The precipitated barium salt was filtered off, washed, and dried in the manner previously described. The yield was practically quantitative.

*Procedure 3*—1.40 gm. of barium *d*-galacturonate were treated with 2.50 gm. of phenylhydrazine hydrochloride, 6.0 gm. of barium acetate, 100 cc. of water, and 3 cc. of glacial acetic acid after the

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with the product that we had introduced in our first publication (20). They have likewise failed to realize that the intermediate *Pectolsäure* which they go to the trouble to prepare from this pectin is essentially the polygalacturonide that we recommended as the starting material in our second publication (19). This polygalacturonide can be obtained commercially at a fraction of the cost of laboratory preparation.

procedure of Goldschmiedt and Zerner (38). The yellow precipitate obtained was filtered off, extracted with ether in a continuous extractor, and finally dried in the usual manner. 1.0 gm. of the hydrazone barium salt was obtained, which is equivalent to 53 per cent of that required theoretically.

*Melting Point*—The compound chars but does not melt or decompose with evolution of gas below 250°.

<i>Analysis</i> —Calculated for $(C_{12}H_{16}O_6N_2)_2Ba$ .		Ba	19 53,	N	7 96
Found.	Procedure 1.	"	19 46,	"	8 08
	" 2.	"	20 01,	"	8 06
	" 3.	"	19 64,	"	8 31

(b) *Preparation of Phenylhydrazine Phenylhydrazone d-Galacturonate (33)*—8.2 gm. of phenylhydrazine were dissolved in 8.2 gm. of 50 per cent acetic acid and 50 cc. of water. To the hydrazine reagent 4.50 gm. of *d*-galacturonic acid dissolved in 25 cc. of water were then added. Precipitation began within 10 to 15 minutes and after standing overnight in the ice chest the impure salt was filtered off and washed with cold water and 30 per cent ethyl alcohol. To purify the compound it was dissolved in 50 per cent ethyl alcohol and the solution clarified with activated blood charcoal. It was then allowed to crystallize from the alcoholic solution. The freshly prepared crystals possessed a slight yellow color. The yield was 5.5 gm. or 60 per cent of that required for a theoretical yield.

*Melting Point*—The derivative melted at 133–134° (uncorrected) with decomposition.

*Rotation*— $[\alpha]_D^{22} = -10.4^\circ \pm 0.5^\circ$  (initial rotation in methyl alcohol,  $c = 1.1$  per cent).

*Analysis*

Calculated for $C_{18}H_{24}O_6N_4$ .		N	14 28,	N. E.*	25 47 cc. 0 1 N alkali
Found.		"	14 33,	"	25 69 " 0 1 " "

\* N. E. represents the neutralization equivalent.

(c) *Preparation of d-Galacturonic Acid Phenylhydrazone*—9.2 gm. of barium phenylhydrazone *d*-galacturonate (Section I, a) were suspended in 30 cc. of 95 per cent ethyl alcohol. To this suspension 35 cc. of 1 N sulfuric acid were added during the course of 20 minutes. The gelatinous mass which formed was then heated on the steam bath for 15 minutes, along with 5 gm. of activated blood charcoal. On cooling the filtered solution, the hydrazone crystal-

lized out in fine yellow needles, which were collected and dried in the usual manner. The mother liquor can be reworked to increase the yield but this effort is not profitable. 2.0 gm. of the hydrazone were obtained which represent 27 per cent of the theoretical yield.

*Melting Point*—The compound melted at 140–141° (uncorrected) with decomposition.

*Rotation*— $[\alpha]_D^{22} = +1.0^\circ \pm 0.5^\circ$  (initial rotation in methyl alcohol,  $c = 1.0$  per cent).

*Analysis*

Calculated for $C_{12}H_{16}O_6N_2$ .	N 9 86,	N. E. 35 20 cc. 0 1 N alkali
Found.	" 9 96,	" 35 31 " 0 1 " "

(d) *Preparation of Phenylhydrazine Phenyllosazone d-Galacturonate (33)*—2.25 gm. of *d*-galacturonic acid and 4.10 gm. of phenylhydrazine were dissolved in 38 cc. of water. After heating for 2 minutes on the steam bath, 4.1 cc. of glacial acetic acid were added to the solution. The reaction mixture was then heated again on the steam bath for 20 to 25 minutes. At the end of this period the precipitate that had formed was filtered off while the solution was still hot. The filtrate was reheated for an additional 30 minutes to induce further precipitation. The second precipitate obtained was combined with the original fraction. The combined crude fractions were dissolved in hot 35 per cent ethyl alcohol, decolorized with activated carbon, and allowed to crystallize. The purified product was obtained as a light yellow semicrystalline mass. For analysis the compound was dried in the previously described manner. Only 0.25 gm. of the derivative was obtained, whereas a theoretical yield requires 5.56 gm.

*Melting Point*—The compound melts at 130–131° (uncorrected) with decomposition.

*Rotation*— $[\alpha]_D^{22} = +2.40^\circ \pm 0.5^\circ$  (initial rotation in 95 per cent methyl alcohol,  $c = 1.0$  per cent).

*Analysis*

Calculated for $C_{24}H_{28}O_8N_6$ .	N 17 50,	N. E. 20 82 cc. 0 1 N alkali
Found.	" 17 00,	" 21 35 " 0 1 " "

(e) *Preparation of Barium Phenyllosazone d-Galacturonate*—The mother liquor that remained after the recrystallization of the

phenylhydrazine phenylosazone *d*-galacturonate (Section I, *d*) was titrated with 0.2 N barium hydroxide to a phenolphthalein end-point and after standing overnight the precipitated barium salt of the osazone was filtered off, washed with carbon dioxide-free water, alcohol, ether, and finally dried in the above described manner. Only 0.5 gm. of the barium salt was obtained by this procedure.

*Melting Point*—The compound chars but does not melt or decompose with evolution of gas below 250°.

<i>Analysis</i> —Calculated for $(C_{18}H_{19}O_6N_4)_2Ba$ .	Ba 15.62, N 12.73
Found.	" 15.42, " 12.84

## II. *p*-Bromophenylhydrazine Derivatives of *d*-Galacturonic Acid

(a) *Preparation of Barium p*-Bromophenylhydrazone *d*-Galacturonate. *Procedure 1*—1.00 gm. of *p*-bromophenylhydrazine was dissolved in a mixture of 12 cc. of warm water and 3.5 cc. of 50 per cent acetic acid. To this reagent a solution of 1.45 gm. of barium *d*-galacturonate and 6.00 gm. of barium acetate in 15 cc. of water was added. Within 10 minutes a heavy granular precipitate settled out which remained unchanged on the addition of 30 cc. of 95 per cent ethyl alcohol. After standing for 3 to 4 hours the precipitate was filtered off, washed successively with water, absolute ethyl alcohol, and ethyl ether. The product was finally dried at 60° over calcium chloride under 15 mm. pressure. The yield was 1.30 gm. or 55.0 per cent of the theoretical amount.

*Procedure 2*—0.50 gm. of *p*-bromophenylhydrazine *p*-bromophenylhydrazone *d*-galacturonate (Section II, *b*) was dissolved in 30 cc. of warm 60 per cent ethyl alcohol. This solution was then titrated with 0.2 N barium hydroxide to a phenolphthalein end-point. The precipitated barium salt was filtered off, washed, and dried in the manner described above. A practically quantitative yield was obtained.

*Procedure 3*—1.40 gm. of barium *d*-galacturonate were treated with 4.00 gm. of *p*-bromophenylhydrazine,<sup>6</sup> 6.00 gm. of barium acetate, 100 cc. of water, and 3 cc. of glacial acetic acid after the procedure of Goldschmiedt and Zerner (38). The yellow precipitate obtained was extracted continuously with ether and then dried in the usual manner. 1.30 gm. of the derivative were obtained which represent 55 per cent of the theoretical yield.

<sup>6</sup> The hydrochloride may be used in place of the free base.

*Melting Point*—No true melting point was observed below 250°.

<i>Analysis</i> —Calculated for $(C_{12}H_{14}O_6N_2Br)_2Ba$ .		Ba	15	95,	N	6	51
Found.	Procedure 1.	"	16	03,	"	6	34
	" 2.	"	15	97,	"	6	53
	" 3.	"	15	62,	"	6	71

(b) *Preparation of p-Bromophenylhydrazine p-Bromophenylhydrazone d-Galacturonate (33)*. Procedure 1—3.00 gm. of *p*-bromophenylhydrazine were dissolved in a mixture of 30 cc. of water and 10.5 cc. of 50 per cent acetic acid. 1.07 gm. of *d*-galacturonic acid dissolved in 5.0 cc. of water were then added to the hydrazine solution. Within 10 minutes precipitation began, and after standing overnight at room temperature the precipitate was filtered off and washed with cold water and a small quantity of cold 70 per cent ethyl alcohol. The product obtained was redissolved in hot 60 per cent ethyl alcohol, the solution decolorized, filtered, and allowed to crystallize. The recrystallized product was isolated in the form of slightly yellow prisms having a tendency to form aggregates. The yield was 1.10 gm. or 36 per cent of that required for a theoretical yield.

*Procedure 2*—1.00 gm. of *d*-galacturonic acid, 3.60 gm. of *p*-bromophenylhydrazine in 3.0 cc. of glacial acetic acid, and 25 cc. of water were heated on the steam bath. A yellow precipitate formed almost instantaneously which persisted on continued heating. After being heated for 30 to 40 minutes the precipitate was filtered off and washed with dilute acetic acid and warm water. The crude product was dissolved in hot 70 per cent ethyl alcohol; the solution decolorized, filtered, and allowed to crystallize. The recrystallized derivative was washed with cold 70 per cent ethyl alcohol and dried at 30° over calcium chloride under 15 mm. pressure. 1.60 gm. of the derivative were obtained which are 56 per cent of the theoretical yield.

*Melting Point*—The compound melted at 145–146° (uncorrected) with decomposition.

*Rotation*— $[\alpha]_D^{22} = +9.0^\circ \pm 2.0^\circ$  (initial rotation in methyl alcohol,  $c = 0.7$  per cent).

*Analysis*

Calculated for $C_{18}H_{21}O_6N_4Br_2$ .		N	10	19,	N. E.	18	18	cc.	0	1	N alkali
Found.	Procedure 1.	"	10	31,	"	17	79	"	0	1	" "
	" 2.	"	10	12,	"	18	48	"	0	1	" "



(c) *Preparation of p-Bromophenylhydrazone of d-Galacturonic Acid*—7.50 gm. of barium *p*-bromophenylhydrazone *d*-galacturonate (Section II, a) were suspended in 20 cc. of water and 40 cc. of 95 per cent ethyl alcohol. 17.5 cc. of *N* sulfuric acid were added in the course of 15 minutes and the reaction mixture heated on the steam bath for 20 to 25 minutes. After the addition of a small amount of blood charcoal the insoluble matter was filtered off. On cooling, the hydrazone of the free acid crystallized out in a short time. It was filtered off, washed with cold 50 per cent ethyl alcohol, and dried at 30° over calcium chloride under 15 mm. of pressure. The yield can be increased by concentrating the mother liquor but the resulting product is usually somewhat colored. The yield was 1.20 gm. or 19.5 per cent of the theoretical yield.

*Melting Point*—The derivative melted at 150–151° (uncorrected) with decomposition.

*Rotation*— $[\alpha]_D^{22} = +11.5^\circ \pm 3.0^\circ$  (initial rotation in methyl alcohol, *c* = 1.36 per cent).

#### *Analysis*

Calculated for  $C_{12}H_{15}O_6N_2Br$ . N 7.72, N. E. 27.54 cc. 0.1 *N* alkali  
 Found. " 7.74, " 27.77 " 0.1 " "

(d) *Preparation of p-Bromophenylhydrazide p-Bromophenylhydrazone of d-Galacturonic Acid*—0.97 gm. of *d*-galacturonic acid, 3.35 gm. of *p*-bromophenylhydrazine hydrochloride, 1.23 gm. of sodium acetate, and 2 cc. of glacial acetic acid were dissolved in a mixture of 10 cc. of water and 15 cc. of pyridine. After the reaction mixture had been heated on the steam bath for 40 minutes, 2 gm. of activated carbon were added and the solution filtered. The filtrate was poured into 400 to 500 cc. of cold water, the precipitate collected and washed copiously with water. The crude product was pressed dry on the filter and suspended in warm 95 per cent ethyl alcohol for a few minutes and again filtered. After being washed with 95 per cent ethyl alcohol, it was dissolved in a small amount of pyridine and precipitated by pouring into 400 cc. of cold water. The purified derivative was filtered off, pressed dry on the funnel, washed with 95 per cent ethyl alcohol, and dried at 60° over phosphorus pentoxide in a vacuum. 0.65 gm. of the derivative was obtained, representing 23.7 per cent of the theoretical yield.

*Melting Point*—The derivative melted at 174–175° (uncorrected) with decomposition.

*Rotation*— $[\alpha]_D^{22} = +17.7^\circ \pm 1.0^\circ$  (initial rotation in pyridine,  $c = 0.8$  per cent).

*Analysis*—Calculated for  $C_{11}H_{11}O_4N_4Br_2$ . N 10.53, Br 30.03  
Found. " 10.62, " 30.00

### III. *p*-Bromophenylhydrazine Derivatives of *d*-Mannuronic Acid

(a) *Preparation of Barium p*-Bromophenylhydrazone *d*-Mannuronate. *Procedure 1*—1.45 gm. of barium *d*-mannuronate and 6.00 gm. of barium acetate were dissolved in 15 cc. of water. A sufficient amount of a 7.5 per cent *p*-bromophenylhydrazine acetate solution was then added so that 1.00 gm. of the free base was present. After shaking for 1 to 2 hours a granular precipitate began to form. The precipitation was complete in 4 hours. 30 cc. of 95 per cent ethyl alcohol were then added and the precipitate filtered off, washed with water, 95 per cent ethyl alcohol, and ethyl ether. The product was finally dried over calcium chloride at 60° under 15 mm. pressure. 1.40 gm. were obtained, representing 59 per cent of that required by theory.

*Procedure 2*—1.40 gm. of barium *d*-mannuronate were treated with 4.10 gm. of *p*-bromophenylhydrazine hydrochloride, 6.00 gm. of barium acetate, 100 cc. of water, and 5 cc. of glacial acetic acid after the procedure of Goldschmiedt and Zerner (38). A yellow precipitate was formed immediately and persisted throughout the prescribed period of heating. The precipitate was filtered off, washed with hot water, 95 per cent ethyl alcohol, and ethyl ether. The preparation was dried as above. 1.30 gm. were obtained whereas the theoretical yield required 2.30 gm.

*Procedure 3*—0.300 gm. of *p*-bromophenylhydrazone *d*-mannuronolactone (Section III, b) was dissolved in 25 cc. of warm (60°) 95 per cent ethyl alcohol and the resulting solution titrated to a phenolphthalein end-point with 0.2 N barium hydroxide. The precipitated barium salt was allowed to stand overnight and was then filtered off, washed, and dried in the usual manner. A practically quantitative yield was obtained.

*Procedure 4*—0.100 gm. of *p*-bromophenylhydrazine *p*-bromophenylhydrazone *d*-mannuronate (Section III, c) was dissolved in

15 cc. of warm 95 per cent ethyl alcohol and converted into the hydrazone barium salt by treatment in the cold with barium hydroxide. A practically quantitative yield was obtained.

*Melting Point*—No true melting point was found below 250°.

<i>Analysis</i> —Calculated for $(C_{12}H_{14}O_6N_2Br)_2Ba$ .		Ba	15 95,	N	6 51
Found.	Procedure 1.		" 15 76,	"	6 36
	" 2.		" 15 99,	"	6 58
	" 3.		" 16 35,	"	6 43
	" 4.		" 15 44		

(b) *Preparation of p-Bromophenylhydrazone d-Mannuronolactone*—0.87 gm. of *d*-mannuronolactone was dissolved in 10 cc. of water and added to a solution of 1.12 gm. of *p*-bromophenylhydrazine hydrochloride and 0.411 gm. of anhydrous sodium acetate in 30 cc. of water. A white crystalline precipitate began to form immediately. It was collected on a filter after the solution was allowed to stand for 3 hours in a closed flask in the ice chest. The crude hydrazone was recrystallized from 40 per cent ethyl alcohol and after being washed with cold 30 per cent ethyl alcohol was dried at 30° over calcium chloride under 15 mm. pressure. 1.00 gm. of the hydrazone was obtained, representing 58.6 per cent of that required for a theoretical yield.

*Melting Point*—The compound melted at 160° (uncorrected) with decomposition.

*Rotation*— $[\alpha]_D^{22} = +64.5^\circ \pm 1.0^\circ$  (initial rotation in methyl alcohol,  $c = 2.3$  per cent).

*Analysis*

Calculated for $C_{12}H_{13}O_6N_2Br$ .		N	8 12,	N. E.	28 98 cc. 0 1 N alkali
Found.		"	8 05,	"	29 09 " 0 1 " "

(c) *Preparation of p-Bromophenylhydrazine p-Bromophenylhydrazone d-Mannuronate*—0.338 gm. of *p*-bromophenylhydrazone *d*-mannuronolactone (Section III, b) and 0.188 gm. of *p*-bromophenylhydrazine were dissolved in 15 cc. of hot 40 per cent ethyl alcohol and heated on the steam bath for 15 minutes. A small quantity of activated charcoal was added to the hot solution and upon filtration the salt crystallized out from the filtrate in colorless aggregates. After filtering off the derivative, it was washed with a small quantity of cold 30 per cent ethyl alcohol and dried at 30° over calcium chloride under 15 mm. pressure. 0.10 gm. of the salt was obtained, whereas 0.54 gm. is required for a theoretical yield.

*Melting Point*—The derivative melted at 143–144° (uncorrected) with decomposition.

*Rotation*— $[\alpha]_D^{22} = +48.5^\circ \pm 1.5^\circ$  (initial rotation in methyl alcohol,  $c = 1.4$  per cent).

*Analysis*

Calculated for  $C_{18}H_{21}O_6N_4Br_2$ . N 10 19, N. E. 18 18 cc. 0 1 N alkali  
Found. " 10 39, " 18 52 " 0 1 " "

(d) *Preparation of p-Bromophenylhydrazide p-Bromophenylhydrazone of d-Mannuronic Acid.* *Procedure 1*—0.88 gm. of *d*-mannuronolactone, 3.35 gm. of *p*-bromophenylhydrazine hydrochloride, and 1.23 gm. of anhydrous sodium acetate were intimately mixed and introduced into a small flask. 40 cc. of boiling water and 1 cc. of glacial acetic acid were then added and the reaction mixture heated on the steam bath for 30 to 40 minutes. A white voluminous precipitate is formed upon the addition of the water, which dissolves after heating for a few minutes. Before the first precipitate has completely dissolved a yellow precipitate begins to appear and continues to form throughout the period of heating. Upon the termination of the heating the precipitate was filtered off and sucked dry. The partially dried, crude derivative was suspended in 15 to 20 cc. of cold 95 per cent ethyl alcohol, filtered off, and washed with a small quantity of cold 95 per cent ethyl alcohol. The product thus obtained was then recrystallized from boiling 95 per cent ethyl alcohol, filtered off, washed with 95 per cent ethyl alcohol and ether, and dried at 30° over calcium chloride under 15 mm. pressure. 0.50 gm. of the derivative was obtained, representing 18.8 per cent of that required for a theoretical yield.

*Procedure 2*—A solid mixture of 0.44 gm. of *d*-mannuronolactone, 1.68 gm. of *p*-bromophenylhydrazine hydrochloride, and 0.61 gm. of anhydrous sodium acetate was treated with 20 cc. of boiling 95 per cent ethyl alcohol and 1 cc. of glacial acetic acid. The reaction mixture was heated on the steam bath for 30 to 40 minutes and then cooled in the ice chest overnight. The crystalline precipitate was filtered off and recrystallized from hot 95 per cent ethyl alcohol. The derivative was washed and dried as described in Procedure 1. 0.60 gm. of the compound was obtained, which is 45.2 per cent of that required for a theoretical yield.

*Melting Point*—The compound melted at 174–175° (uncorrected) with decomposition.

*Rotation*— $[\alpha]_D^{22} = +18.5^\circ \pm 1.0^\circ$  (initial rotation in pyridine,  $c = 0.75$  per cent).

*Analysis*—Calculated for  $C_{12}H_{10}O_6N_4Br_2$ . N 10 53, Br 30 03

Found. Procedure 1. " 10 61, " 30 33

" 2. " 10 40, " 30 30

## DISCUSSION

### *I. Phenylhydrazine Derivatives of d-Galacturonic Acid*

In giving a résumé of the properties of the phenylhydrazine derivatives of *d*-galacturonic acid described in this paper the preparation of the barium phenylhydrazone *d*-galacturonate (Section I, *a*) which can be obtained by three different methods is of unique interest. This compound is readily formed at low temperatures through the reaction of phenylhydrazine with barium *d*-galacturonate (Section I, *a*). It is not only insoluble in all of the commoner organic solvents but its solubility in boiling water is extremely low. The extreme insolubility in hot water is responsible for the insurmountable difficulties encountered in the direct preparation of the barium salt of the phenylosazone of *d*-galacturonic acid from the barium salt of the acid. Thus if an attempt is made to prepare barium phenylosazone *d*-galacturonate by following the procedure that Goldschmiedt and Zerner (38) devised for barium *d*-glucuronate the resulting product will invariably be the barium salt of the hydrazone. The insolubility of the hydrazone barium salt (Section I, *a*) coupled with its ease of formation and ready transformation into a water- and alcohol-soluble crystalline product, *i.e.* the hydrazone of the free acid (Section I, *c*), suggests its potential value for the isolation of *d*-galacturonic acid from complex reaction mixtures.

The phenylhydrazine salt of the phenylhydrazone of *d*-galacturonic acid (Section I, *b*) is readily formed when the free acid is treated with phenylhydrazine (33). Ohle and Berend (33) failed in their attempts to recrystallize it from boiling water. Consequently they could not report the analytical data or physical constants. As shown above, the derivative can be recrystallized readily from 50 to 60 per cent ethyl alcohol to yield well defined crystals. This derivative of *d*-galacturonic acid possesses a sharp melting point, and is distinctly levorotatory. In addition it is

soluble in methyl and ethyl alcohol. Its solubility in the common solvents is, however, rather limited at low temperatures.

As pointed out above, the phenylhydrazone of *d*-galacturonic acid (Section I, *c*) can be prepared readily by decomposition of the barium salt (Section I, *a*) with dilute sulfuric acid. It can be obtained in a well defined crystalline form and consequently is more suitable for purposes of identification than the hydrazone barium salt (Section I, *a*). The latter compound is, on the other hand, more readily isolated from reaction mixtures because of its great insolubility.

The phenylhydrazine salt of the phenylosazone of *d*-galacturonic acid (Section I, *d*) reported by Ohle and Berend (33) had a melting point of 140°, whereas the same compound prepared by us melted at 130–131°. Attempts to recrystallize it from hot aqueous ethyl alcoholic mixtures led to higher values for the melting point (144–145°). However, as the melting point of the preparation increased the neutralization equivalent<sup>7</sup> decreased. This possibly indicates that the procedure employed for its purification induced the formation of a hydrazide. Because of this difficulty and in view of the low yield its use can be considered unsatisfactory for either isolation or characterization purposes. While the barium salt of the phenylosazone (Section I, *e*) is more readily isolated than the phenylhydrazine salt (Section I, *d*), the former possesses none of the qualities requisite for purposes of characterization. Furthermore it (Section I, *e*) is inferior to the barium salt of the phenylhydrazone (Section I, *a*) for problems involving the isolation of *d*-galacturonic acid. These features greatly diminish its value for the identification of *d*-galacturonic acid.

## II. *p*-Bromophenylhydrazine Derivatives of *d*-Galacturonic Acid

The outstanding features of the *p*-bromophenylhydrazine derivatives of *d*-galacturonic acid, in contrast to the corresponding phenylhydrazine derivatives (see above), are both their stability and their solubilities, which permit ready purification. In general when small quantities of *d*-galacturonic acid are to be isolated and characterized, the use of *p*-bromophenylhydrazine is more practical than phenylhydrazine. It is difficult for us to understand

<sup>7</sup> Titrated with 0.01 N sodium hydroxide in cold methyl alcohol.

how Ehrlich could have overlooked these facts since he made a special point to the effect that *d*-galacturonic acid, in contrast to *d*-glucuronic acid, would not form insoluble derivatives of *p*-bromophenylhydrazine comparable to those of *d*-glucuronic acid (40).

The barium *p*-bromophenylhydrazone *d*-galacturonate (Section II, *a*) is produced under the same conditions which lead to the formation of the corresponding phenylhydrazine compound. The derivative is insoluble in all of the common solvents and consequently its value lies solely in its application to isolation problems. The Goldschmiedt and Zerner procedure for the preparation of barium *p*-bromophenylosazone *d*-glucuronate (38) when applied to barium *d*-galacturonate will not yield the barium *p*-bromophenylosazone *d*-galacturonate, but will invariably form the *p*-bromophenylhydrazone barium salt (Section II, *a*). The Goldschmiedt and Zerner conditions likewise did not yield the corresponding phenylosazone of barium *d*-galacturonate (see above).

The *p*-bromophenylhydrazine salt of *d*-galacturonic acid *p*-bromophenylhydrazone (Section II, *b*) is probably one of the most desirable derivatives for isolation and characterization purposes. The compound possesses a melting point in a desirable range, has a definitely distinguishable dextrorotation, and possesses solubility characteristics that permit easy purification. Of the two procedures presented, Procedure 2 (Section II, *b*) gives a better yield, but in some cases this factor may be sacrificed when heating might tend to form hydrazine derivatives of accompanying substances. Thus, in the presence of glucose, galactose, or arabinose Procedure 1 (Section II, *b*) would be decidedly more advantageous.

The barium salt of the *p*-bromophenylhydrazone (Section II, *a*) is difficultly soluble and therefore readily isolated. Its high melting point, on the other hand, limits its value. However, with sulfuric acid it is easily converted into the hydrazone of the free acid (Section II, *c*) which may be characterized readily by its melting point, rotation, and neutralization equivalent. Because of the low yields that are obtained the hydrazone of the free acid (Section II, *c*) is not as practical a derivative as the hydrazine salt of the hydrazone (Section II, *b*).

The *d*-galacturonic acid *p*-bromophenylhydrazide *p*-bromophenylhydrazone (Section II, *d*) may find application where the hydrazine compounds of accompanying substances are readily

soluble in 95 per cent ethyl alcohol. This derivative of galacturonic acid is but slightly soluble in warm 95 per cent ethyl alcohol, but is readily soluble in pyridine. It is practically inert to cold or warm dilute alkali. This property indicates the validity of the structure assigned since the osazone of the free acid or of the lactone would react with alkali under the above conditions. In general the hydrazide-hydrazone (Section II, *d*) is inferior to the hydrazine salt of the hydrazone (Section II, *b*) because of lower yields, and greater difficulty of preparation. An attempt was made to prepare a *p*-bromophenylosazone derivative, but in all cases the resulting products were hydrazones contaminated with highly colored impurities.

### III. *p*-Bromophenylhydrazine Derivatives of *d*-Mannuronic Acid

In view of the insolubility and stability of *d*-mannose phenyl- and *p*-bromophenylhydrazone (42, 44), it was not surprising to find that the corresponding *d*-mannuronic acid derivatives possessed similar properties. The barium *p*-bromophenylhydrazone *d*-mannuronate (Section III, *a*) is readily formed by Procedure 1 at room temperature and is insoluble in all of the common solvents. Like the corresponding derivative of *d*-galacturonic acid (see above) the hydrazone barium salt is suitable only for purposes of isolation. It is of interest to point out that barium *d*-mannuronate, when treated according to the procedure of Goldschmidt and Zerner for the preparation of barium *p*-bromophenylosazone *d*-glucuronate (42), will invariably yield the hydrazone barium salt.<sup>8</sup>

The *p*-bromophenylhydrazone of *d*-mannuronolactone (Section III, *b*) is an exceedingly useful derivative. It is readily formed at room temperatures and is insoluble in water. Purification can readily be accomplished by recrystallization from 40 per cent ethyl alcohol. Its melting point is within the desirable range and it is strongly dextrorotatory. The high rotation of the *p*-bromophenylhydrazone of *d*-mannuronolactone (Section III, *b*), and *p*-bromophenylhydrazine *p*-bromophenylhydrazone *d*-mannuronate (Section III, *c*) serves to distinguish these compounds from the corresponding derivatives of *d*-galacturonic acid (see above).

<sup>8</sup> Barium *d*-mannuronate and phenylhydrazine under these conditions also form the barium salt of the phenylhydrazone (unpublished data of the authors).



The *p*-bromophenylhydrazine salt of *d*-mannuronic acid *p*-bromophenylhydrazone (Section III, *c*) will form when free *d*-mannuronic acid is present in solution. Thus in many cases the treatment of a crude product, *e.g.* a mixture of *d*-mannuronic acid lactone and *d*-mannuronic acid, will yield this derivative contaminated with the hydrazone of the lactone. In such cases it is desirable to convert the mixture completely into the hydrazine salt by heating with a sufficient amount of a 40 per cent alcohol solution of *p*-bromophenylhydrazine. The hydrazine salt of the hydrazone (Section III, *c*) possesses all of the desirable qualities shown by the hydrazone of the lactone (Section III, *b*) and, as pointed out above, is easily distinguished from the corresponding compound of *d*-galacturonic acid. The *p*-bromophenylhydrazide of *d*-mannuronic acid *p*-bromophenylhydrazone (Section III, *d*) is readily prepared and easily purified. The physical constants are well within an easily discernible range and suggest the value of this derivative for both isolation and characterization purposes.

In general all of the *p*-bromophenylhydrazine derivatives of *d*-mannuronic acid described herein are suitable for the identification of this acid. Several attempts to prepare the *p*-bromophenyl-osazone led to intractable products which could not be purified.

#### SUMMARY

The preparation and properties of the following derivatives of *d*-galacturonic and *d*-mannuronic acid have been described: Section I, barium phenylhydrazone *d*-galacturonate (*a*), phenylhydrazine phenylhydrazone *d*-galacturonate (*b*), *d*-galacturonic acid phenylhydrazone (*c*), phenylhydrazine phenylosazone *d*-galacturonate (*d*), barium phenylosazone *d*-galacturonate (*e*); Section II, barium *p*-bromophenylhydrazone *d*-galacturonate (*a*), *p*-bromophenylhydrazine *p*-bromophenylhydrazone *d*-galacturonate (*b*), *d*-galacturonic acid *p*-bromophenylhydrazone (*c*), the *p*-bromophenylhydrazide *p*-bromophenylhydrazone of *d*-galacturonic acid (*d*); Section III, barium *p*-bromophenylhydrazone *d*-mannuronate (*a*), *p*-bromophenylhydrazone *d*-mannuronolactone (*b*), *p*-bromophenylhydrazine *p*-bromophenylhydrazone *d*-mannuronate (*c*), and the *p*-bromophenylhydrazide *p*-bromophenylhydrazone *d*-mannuronic acid (*d*). Their value as suitable derivatives for purposes of isolation and identification has been discussed.

The behavior of barium *d*-galacturonate and barium *d*-mannuronate in the Goldschmiedt and Zerner reaction has been pointed out for the first time.

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# STUDIES IN THE NUTRITIONAL ANEMIA OF THE RAT

## VII. INFLUENCE OF PARENTERALLY ADMINISTERED IRON\*

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In the treatment of anemia with orally administered iron, the question of absorption is one that is often raised when unsatisfactory results are obtained clinically. For this reason, iron has sometimes been given parenterally when prompt results were sought.

In their first study on nutritional anemia, Mitchell and Vaughn (1) correlated the antianemic potency of a number of iron salts with their solubility, hence, presumably with their absorbability. It is conceivable, therefore, that the conflicting results reported on the value or ineffectiveness of pure iron salts in curing nutritional anemia might be explained on the basis of good or poor absorption of iron.

For these reasons it was deemed advisable to attempt the circumvention of the problem of intestinal absorption—a question that is difficult to solve experimentally—by parenteral administration of the iron. We present in this paper experimental data on the treatment of nutritional anemia in the rat by intraperitoneal injection of copper-free salts of iron. It has been found that very

\* A preliminary report of this work was presented before the Section on Experimental Medicine of the Cleveland Academy of Medicine, January 8, 1932.

The data are taken in large part from a thesis submitted by Margaret W. Eveleth to the Graduate School of Western Reserve University, June, 1932, in partial fulfilment of the requirements for the degree of Master of Science.

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small amounts of pure iron, parenterally administered, markedly stimulate growth in body weight and red blood cell production, and the proper dosage results in the restoration of the hemoglobin to the normal level. Furthermore, the rate of erythropoiesis is not significantly affected by the addition of small amounts of copper to the iron, even when precautions are observed to minimize the storage of copper in the rats prior to the experimental period.

#### EXPERIMENTAL

Young rats were made anemic in the manner previously described (2), by removal from their mothers when about 3 weeks of age and restriction to a diet of raw cow's milk (certified). A group of animals was also treated according to the technique of Elvehjem and Kemmerer (3), slightly modified, in an effort to prevent the storage of substances that might be obtained from the stock diet. We found it advisable not to remove the mother from the cage, for separate feeding with stock rations, until the young were 10 days old. In agreement with Elvehjem and Kemmerer (3), we found it possible to diminish materially the time required to bring the blood of the animals to the required anemic level.

Portions of the milk were removed from the daily supply; the samples were pooled and analyzed at intervals. The copper content, determined by the Biazzo method, as described by Ansbacher, Remington, and Culp (4), ranged from 0.30 to 0.38 mg. and averaged 0.34 mg. per liter for seven separate determinations.

The ferric chloride solution was prepared from pure electrolytic iron and was subsequently treated to remove possible copper contamination. The solution was diluted with warm, sterile saline solution (prepared from copper-free sodium chloride) and injected into the peritoneal cavity immediately after dilution with a tuberculin syringe and chromium-plated needle. The dilutions were arranged so that the amount injected into each animal was always 0.50 cc. regardless of the iron dosage. The acidity of the original stock solutions was also adjusted so that the reaction of the material injected was between pH 2.0 and 2.5. To avoid too much irritation the animals received injections only on alternate days.

At regular weekly intervals hemoglobin determinations, carried out by the benzidine method (5), were made upon blood obtained

from the tail. At the same time the red blood cells were enumerated in a Levy-Neubauer chamber, isotonic saline solution being adopted as the diluting fluid.

The results of the first experiments are recorded in Table I, where, for the sake of brevity, only a few of the figures are pre-

TABLE I

*Stimulation of Growth, Erythropoiesis, and of Hemoglobin Production with Varying Dosages of Injected Iron*

The figures represent the averages for two animals.

Dosage of Fe every other day		Weeks				
		0	4	7	14	22
mg						
0 025	Weight, gm.	67	113	147	210	231
	R.b.c., millions	2 3	5 6	7 5	11 1	11 5
	Hb, gm. per 100 cc.	2 3	2 7	4 3	6 8	7 9
0 050	Weight, gm.	80	128	162	220	220
	R.b.c., millions	2 7	5 5	7 9	13 2	13 8
	Hb, gm. per 100 cc.	2 7	3 2	4 5	8 2	8 1
0 100	Weight, gm.	64	106	149	212	231
	R.b.c., millions	2 3	8 7	8 8	12 5	11 8
	Hb, gm. per 100 cc.	2 8	4 9	5 3	8 8	9 8
0 200	Weight, gm.	96	149	188	213	
	R.b.c., millions	3 3	8 7	11 8	13 4	
	Hb, gm. per 100 cc.	2 5	6 2	8 5	14 3	
0 500	Weight, gm.	79	149	195		
	R.b.c., millions	3 5	10 6	11 9		
	Hb, gm. per 100 cc.	1 6	11 0	13 5		
1 000	Weight, gm.	82	114	136		
	R.b.c., millions	3 5	10 4	8 4		
	Hb, gm. per 100 cc.	2 2	11 6	8 0		
1.500	Weight, gm.	72	104	126		
	R.b.c., millions	3 6	8 7	9 4		
	Hb, gm. per 100 cc.	2 9	11 6	14 1		

sented. The rats had been rendered anemic by the method of Waddell *et al.* (6), as used in this laboratory (2). The study of the blood picture and growth curve prior to the experimental period showed the development of anemia and the gradual decline in the rate of growth, many of the animals losing weight in the last 1 or 2 weeks of the period of depletion. As evidenced in Table I,

the injection of iron resulted in a marked increase in body weight and the indefinite prolongation of life with even the smallest dosage. It is apparent that intraperitoneally injected iron was utilized by these anemic rats. The rate of growth was identical with all dosages used. The extent of the growth, incidentally, indicates that the milk was of satisfactory vitamin content. The rats consumed 20 to 30 cc. of milk daily at the beginning of the experiment, increased the intake with the institution of iron therapy, and drank as much as 90 to 100 cc. per day after attaining a weight of about 125 gm.

Even had the hemoglobin level of the blood remained constant, it is evident that a substantial increase in total hemoglobin of the body must occur when animals more than treble their weight. All the rats, in addition to the increase in total hemoglobin that must accompany growth, were also able to raise the hemoglobin concentration of the blood to an extent depending upon the dosage of iron. With 0.5 mg. or more of iron the hemoglobin reached the normal level in 7 weeks or less; with 0.2 mg., the hemoglobin regeneration proceeded more slowly. Lower dosages did not result in complete restoration within 5 months, at which time the experiment was discontinued.

The production of red blood cells was quickly stimulated by all dosages of iron. The normal level (about 9.0 million red cells per c.mm.) was reached and exceeded in nearly all cases. With dosages of 0.025, 0.050, and 0.100 mg. of iron respectively, a definite *polycythemia accompanied by low hemoglobin* was evidenced after 11 weeks of injections and was maintained throughout the experimental period. With larger amounts of iron the normal erythrocyte level was attained and exceeded much more quickly.

After the conclusion of the experimental period the animals were sacrificed and examined for possible injury from the injected iron. No inflammation or other gross signs of toxicity were noted. However, it was apparent from the behavior of the animals that dosages of 0.500 mg. or more of iron were not well tolerated by the rats. Immediately following injections of these amounts the rats became hyperirritable. They would move about quickly, dragging the hind leg on the side of the injection, and then after a time become unusually quiet. The two rats receiving 1.00 mg. of iron attained the normal hemoglobin level in about 5 weeks,

and then declined. The blood findings of the animals receiving 1.500 mg. of iron also showed erratic fluctuations, although these variations do not show in the outline of our data as presented in Table I. Two rats which weighed slightly less than 50 gm. each died within a few hours after receiving a single injection of 2.0 mg. of iron. From these results it was concluded that 0.500 mg. of iron represents a border line dosage, greater amounts being toxic and lesser quantities producing a slow but regular increase in blood hemoglobin.

TABLE II

*Influence of Injected Iron, Both with and without Added Copper, on Hemoglobin Production*

Dosage of Fe every other day  mg *	Rats receiving only Fe intraperitoneally						Rats receiving Fe intraperitoneally + Cu					
	No of rats	Average gain in weight in 6 wks †	Average Hb		Average R b c		No of rats	Average gain in weight in 6 wks †	Average Hb		Average R b c	
			Begin-ning	End	Begin-ning	End			Begin-ning	End	Begin-ning	End
gm	gm	gm.	mil-lions	mil-lions	gm	gm	gm.	mil-lions	mil-lions			
0 025	7	53	2 4	3 9	2 9	6 6	2	42	1 8	4 0	3 8	8 4
0 050	7	56	2 7	3 9	2 5	7 1	5	67	2 4	4 5	3 6	9 3
0 100	7	59	2 4	6 1	2 5	9 7	5	73	2 4	6 8	3 2	11 2
0 200	7	70	2 6	8 1	3 0	10 4	4	76	2 3	8 8	3 4	11 5
0 500	5	90	2 0	10 6	3 3	9 8	2	92	2 5	8 8	2 8	8.0

\* The rats averaged 66 gm. in weight at the start of the experimental period.

Several rats from this and subsequent groups were selected for histological study of some of the organs. No pathological changes<sup>1</sup> were evident except a fatty parenchymatous degeneration of the liver in all rats examined. Since the same condition was found in anemic rats that had never been injected with iron, the condition was not due to the treatment. This fact may indicate that hitherto unsuspected defects, that are not improved by measures promoting a regeneration of hemoglobin, may be produced in rats by restriction to a diet of cow's milk. Some of the pathological

<sup>1</sup> We are indebted to Dr. Harry Goldblatt of the Institute of Pathology for these histological examinations.



changes resulting from a milk diet have been emphasized by Schmidt (7).

Further experiments were performed to test the effect of added copper on the regeneration of hemoglobin produced by the parenteral administration of pure iron. In these experiments rats were rendered anemic by the technique previously described and also by the slightly modified method of Elvehjem and Kemmerer (3). This, it will be noted, consists essentially in the limitation of the food of the young rat to rat milk and cow's milk, while precautions were taken to prevent coprophagy. Because no differences in response were evident, the data obtained with both

TABLE III

*Response of Anemic Rats Fed on Milk Containing 0.14 Mg of Copper per Liter*

Treatment*	No of rats	Average weight		Average Hb		Time of observation
		Beginning	End	Beginning	End	
		gm	gm	gm	gm	wks
Fe + Cu by mouth	2	33	123	2 1	12 1	7
" by mouth	2	36	93	2 2	9 5	7
" " "	3	50	89	2 8	5 9	4
" parenterally	1	36	117	3 3	7 2	7
" "	2	43	79	1 8	7 2	2½

\* The dosage of iron was 0.50 mg. daily by mouth or on alternate days intraperitoneally. The dosage of copper was 0.025 mg per day by mouth, when administered.

methods have been treated together, and the average figures are presented in Table II. The copper was administered simultaneously with the iron, and was either injected or fed. The dosage consisted of 0.025 mg. of copper as copper sulfate every other day. The experimental period was limited to 6 weeks. The blood was examined at weekly intervals, but only the average values for the beginning and end of the experimental period are included in Table II. None of the rats died during the experiment. All gained in weight. In many of the rats the red blood cells attained the normal level and in some cases exceeded it. The data for the cell counts are presented to show the marked stimulation in erythrocyte production that was obtained with even the smallest

dosage of iron. The hemoglobin response was practically identical, within biological variations, in the animals that received iron and those that received iron and copper.

The fact that added copper does not accelerate the rate of recovery obtainable with injected iron is a point worthy of emphasis. Beard and Myers (2) found that increased dosages of orally administered iron accelerated the rate of recovery of anemic rats. When 2 mg. of iron were fed daily, the rate of hemoglobin production was fully as rapid as it was when smaller, but adequate doses of iron were administered in conjunction with copper. One is thus led to conclude that the amount of absorbed iron is the important factor in hemoglobin production.

Although the above results show definitely the failure of added copper to accelerate the recovery of rats from nutritional anemia, they cannot be interpreted to prove copper is not essential, since the milk contained 0.34 mg. of copper per liter. In subsequent experiments milk subjected to the minimum of handling was used. This was achieved through the kindness of Dr. Donald F. Eveleth, who, throughout the experiment, personally milked a cow directly into a Pyrex flask and brought the milk to the laboratory. The cow was a Guernsey, fed on hay and a small amount of bran. The milk contained 0.14 mg. of copper per liter by analysis, this figure being the average of six separate determinations (range 0.13 to 0.16 mg.). All rats were rendered anemic by the modified technique of Elvehjem and Kemmerer. Some animals received injections of 0.500 mg. of iron on alternate days, others were given 0.500 mg. enterally each day, and one group received daily 0.025 mg. of copper by mouth, in addition to the iron. The rate of recovery for inexplicable reasons was slow, even with copper additions. Unfortunately, owing to the removal of one of us (M. W. E.) the experiments had to be discontinued before complete regeneration of hemoglobin had been attained. They are being repeated and extended at the present time. As preliminary evidence, however, the data presented in Table III show an unmistakable increase in hemoglobin produced by iron alone, either injected or fed.

#### DISCUSSION

After the present series of experiments had been completed, there appeared the paper by Keil and Nelson (8) on the value of

intraperitoneally injected iron in the treatment of nutritional anemia. Regarding the evidence presented by Keil and Nelson, purporting to show the effect of injections of hydrochloric acid on the maintenance of the hemoglobin concentration of the blood, we cannot believe the acidity of our injected solutions played any significant rôle in the recovery of the anemic animals.

Keil and Nelson (9) found that rats fed on milk containing 0.24 mg. of copper per liter, estimated by the carbamate method, did not recover with iron alone, unless it was injected intraperitoneally. Our results show that recovery from anemia occurs with pure iron, administered either by mouth or parenterally, when milk which contains 0.34 mg. of copper per liter is employed. Although the experiments could not be prolonged sufficiently to make the data entirely conclusive, the results with milk containing 0.14 mg. of copper per liter show that considerable hemoglobin production is possible when such milk is supplemented with pure iron. Could the different experiences of our laboratory, as compared with the results obtained in other localities, be due to variations in the utilization of iron administered by mouth? The efficacy of intraperitoneally injected iron, as observed by Keil and Nelson and ourselves, indicates that such might be the case. It is true that Elvehjem and Sherman (10), on the basis of analyses of the liver and spleen for iron, conclude that copper has no effect on the assimilation of iron. Further experimental evidence, however, will be necessary before all the factors influencing the absorption and utilization of iron are understood.

In its absorption and excretion iron would appear to resemble calcium in many respects. Like calcium, by far the larger proportion is excreted by way of the intestine. We might judge from this that it was not only absorbed with difficulty, but that, considering the very small renal excretion, the intestine was the chief path for excretion after absorption. Since vitamin D is known to play a vital, though not well understood, rôle in calcium absorption and utilization, may it not be that some similar factor plays a vital rôle in the absorption and utilization of iron? It is of interest in this connection that ultra-violet light has been claimed to exert a favorable influence upon red blood cell and hemoglobin formation (11).

From the experiments of Hart and his collaborators it may be

calculated that their anemic rats die on milk containing added iron and sufficient copper to furnish approximately 0.0050 mg. of copper per day, but, according to their data, the same rats thrived if the total copper intake per day was increased to 0.0075 mg. Lintzel (12) and Beard and Myers (2), in reviewing this work, have asked the seemingly pertinent question why the copper already present in the milk (equal to at least twice the minimum effective dose of copper, according to present methods of analysis) should have absolutely no effect. In this laboratory we have exercised due precautions to avoid copper contamination of the milk and of the iron supplements, or the storage of excessive iron or copper in the tissues of the rats prior to the experimental regimen, and the animals have recovered from anemia with pure iron salts.

Beard and Myers (13) were originally criticized by Waddell, Steenbock, and Hart (14) on the basis that the Fe they employed was contaminated with Cu. Beard and Myers (2) discussed the fallacies in this criticism, pointing out that the amount of Cu which would necessarily have had to be present in the Fe to merit this criticism was practically impossible. They suggested that the milk was a more probable source of difficulty. In a recent paper by Elvehjem and Hart (15), these authors now attribute the success of the experiments of Beard and Myers with iron alone to the milk they employed, without acknowledging that this possibility was first mentioned by Beard and Myers.

#### SUMMARY

Rats made anemic by restrictions to a ration of raw cow's milk (certified) received intraperitoneal injections of pure iron. Precautions were taken to limit the acidity of the iron solutions, and the injections were made every other day. Dosages above 0.5 mg. of iron were not well tolerated, although they resulted in stimulation of hematopoiesis.

Complete regeneration of hemoglobin was obtained in 7 weeks with a 0.5 mg. dosage of iron, injected on alternate days. Smaller amounts (0.2, 0.1, 0.05, and 0.025 mg.) produced only partial recovery of hemoglobin, but did completely restore the red blood cells to the normal level and above.

It is significant that the animals receiving insufficient amounts

of iron exhibited a constant polycythemia (above 11.0 million red cells per c.mm. of blood) after 11 weeks on the regimen. This was coincident with a subnormal hemoglobin concentration (less than 10 gm. per 100 cc. of blood) persisting throughout the remainder of the experimental period.

The addition of copper to the parenterally administered iron, in doses of 0.025 mg. every other day either orally or intraperitoneally, did not significantly accelerate the rate of hemoglobin production, red cell formation, or growth in body weight.

The results show that the intraperitoneal injection of iron is particularly effective in curing the nutritional anemia of the rat, and suggest that absorption may play a leading rôle in the utilization of iron for hemoglobin production.

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## STUDIES IN THE NUTRITIONAL ANEMIA OF THE RAT

### VIII. A METHOD FOR THE ESTIMATION OF HEMOGLOBIN AND ERYTHROCYTES ON A SINGLE SMALL SAMPLE OF BLOOD\*

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In studies of hemoglobin production the determination of the number of erythrocytes is quite as important as the estimation of the blood pigment itself. The number of methods for the evaluation of the red cell count as well as the hemoglobin concentration is legion. Most of the older methods have been reviewed by Bürker (1) and many new procedures have since been described. Though there would seem to be a technique suitable for any special conditions, the procedure described herein can be used with advantage in experimental work with small animals. It consists in the utilization of the suspension remaining in the dilution pipette, after the determination of the cell count, for the estimation of hemoglobin by the benzidine method. Only ordinary equipment is necessary and the method has proved both practical and sufficiently accurate.

#### *Method*

A drop of blood is drawn into an ordinary red blood cell-counting pipette to the 0.5 volume mark and diluted to the line marked 101 volumes with isotonic saline (0.9 per cent NaCl solution).<sup>1</sup>

\* Presented in demonstration before the Twenty-sixth meeting of the American Society of Biological Chemists at Philadelphia, April 29, 1932.

† Crile Scholar.

<sup>1</sup> Occasionally we have noted a slow hemolysis in saline suspensions of blood from anemic rats. This phenomenon may be due to the impurity found by Williams and Jacobs (2) to be regularly toxic to the erythrocytes of teleost fishes. We have not observed the destruction of cells in normal rat blood, nor in anemic blood if the diluting fluid was prepared from a sample of Baker's c.p. NaCl that was available in the laboratory.

After this is shaken for at least 2 minutes a drop of the suspension is placed in the Levy-Neubauer counting chamber and the red cells enumerated (3). The remainder of the fluid in the pipette is immediately transferred to a small watch-glass. With the minimum of delay, to avoid errors due to the sinking of the corpuscles, 0.1 cc. of the suspension is removed with a calibrated pipette and added to 2 cc. of benzidine reagent previously placed in a test-tube graduated at 25 cc. To the contents of the tube 0.9 cc. of water is added and mixed, followed by 1 cc. of 0.6 per cent  $\text{H}_2\text{O}_2$ . The color is allowed to develop for at least 100 minutes, then diluted, and compared with a standard diluted blood similarly treated (4). If only one or two determinations are to be made, it is permissible to dilute the contents of the tubes after 20 or 30 minutes and to compare the colors with a standard allowed to develop for exactly the same length of time. It is advisable to make duplicate determinations of the hemoglobin concentration; there is ample material for doing this with the blood-counting pipettes available on the market at the present time.

For making the dilution of the blood suspension it is convenient to have a precision pipette constructed by sealing a Folin 0.1 cc. and an Ostwald 1 cc. pipette to a 3-way stop-cock.<sup>2</sup> The capacity of the lower part of the pipette is adjusted to contain exactly 0.100 cc. from the polished tip to the bottom of the stop-cock. The pipette is also calibrated to deliver 1 cc. from a mark above the bulb of the Ostwald pipette, through one bore of the cock to the tip. In practice, the pipette is first filled with water to the upper mark, the cock is turned, and the lower portion drained and dried. One can then secure the 0.1 cc. sample of blood suspension and transfer it with 0.9 cc. of water into the reaction tube quickly and accurately.

The determination of hemoglobin is thus made upon blood diluted 2000 times, multiplied by the correction factors of the two pipettes. It is essential that all glassware involved in measuring the blood should be calibrated. It may be mentioned that even the red cell dilution pipettes certified by the United States Bureau of Standards have an allowable error of  $\pm 5$  per cent. For the purpose of enumeration of red cells, this tolerance is probably as

<sup>2</sup> A precision pipette, as described, was made for us by Mr. James D. Graham of the University of Pennsylvania, Philadelphia.

rigid as is necessary, but for the combined count and pigment determination the dilution afforded by the pipette must be known more precisely. The calibration can be made colorimetrically with sufficient accuracy, a sample of fresh oxalated blood being used (5). The blood is drawn to the mark on the pipette to be calibrated and transferred quantitatively into a suitable known volume of water. A dilution of about 2000 times is satisfactory. The hemoglobin concentration is determined with the benzidine method, a standard prepared from the same blood and of accurately known dilution, such as 1 cc. diluted to 2 liters, being used for comparison.

With a Malassez pipette calibrated at the 0.2, 0.4, 0.5, 0.6, 0.8, 1.0, and 101 marks, and a single sample of oxalated blood from a

TABLE I  
*Reproducibility of Results with Present Method*

Amount of blood	Red cells	Hemoglobin
cc	millions per c mm	gm per 100 cc
0 0022	3 40	10 9
0 0044	3 40	10 7
0 0056	3 36	10 7
0 0067	3 42	10 8
0 0089	3 45	10 6
0 0111	3 30	10 6

slightly anemic subject, the series of values presented in Table I was obtained. The figures represent average values of quadruplicate determinations for each dilution. They show the flexibility of the method over a wide range of concentrations and the accuracy attainable.

The present method requires careful technique to secure the best results. While it is primarily adapted for experimental work involving a large number of blood examinations, it should also prove of value for the determination of the color index of finger tip blood, because of the ease with which samples may be secured and transported to the laboratory.

#### SUMMARY

A method is described whereby the estimation of both red blood cells and hemoglobin may be carried out on the 0.005 cc. of blood



withdrawn for the red cell count. The accuracy of the hemoglobin estimation depends largely upon the accuracy with which the blood can be measured. With care, duplicate determinations should agree within 2 or 3 per cent.

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## THE ANTIMONY TRICHLORIDE REACTION WITH COMPOUNDS CONTAINING FIVE-MEMBERED MONO-HETEROCYCLIC RINGS

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Rosenheim and Drummond (1) in 1925 described a color test given by cod liver oil upon interaction with arsenic trichloride. Carr and Price (2) introduced some modifications in the procedure adopted by Rosenheim and Drummond and substituted antimony trichloride for the arsenic compound. The color obtained with these reagents was taken to indicate the presence of vitamin A.

The Carr-Price reaction has been employed in the colorimetric method for the quantitative determination of vitamin A in cod liver oil. Several investigators (3-12), introducing various modifications, have reported agreement between the biological method for the estimation of the vitamin and the chemical method involving the production of color with antimony trichloride and its subsequent measurement in the Lovibond tintometer. Other investigators (13-18), however, could not find any close correlation between the chemical and the biological method. By employing the unsaponifiable residue instead of the oil itself, Andersen and Nightingale (19), Norris and Church (6), Smith and Hazley (20), and also Coward, Dyer, Morton, and Gaddum (21), claim to have obtained reliable values for vitamin A content.

The antimony trichloride color test is hardly to be considered specific for vitamin A, although it may serve to establish some chemical relation between this vitamin and the type of compounds giving positive reactions. Sterols also have been reported by Wokes (22), by Heilbron and Spring (23), and by Seel (24) to give characteristic color reactions with antimony trichloride. Among the sterols may be mentioned phytosterol, cholesterol, ergosterol,

and their various derivatives. Heilbron and Spring maintain that sterols giving a positive reaction contain the  $\Delta^{12}$  or  $\Delta^{13}$  ethenoid linkage. The absorption spectra of the various reaction mixtures have not as yet been reported.

Carotenoid pigments also give characteristic colors with antimony trichloride (25-28). Among those reported as reacting positively are carotene, diiodocarotene, bixin, capsanthin,  $\alpha$ -crocetin, dihydro- $\alpha$ -crocetin, dihydroisonorbixin, fucoxanthin, lutein, lycopin, and zeaxanthin. The belief has been ventured that the antimony trichloride becomes attached to one of the conjugated double bonds in the carotenoid molecule. The color reaction differs in intensity with the change in the polyene groupings and is in all probability influenced by other portions of the molecule.

We wish to report new types of compounds that give characteristic color reactions with antimony trichloride. These types comprise the 5-membered monoheterocyclic rings, thiophene, pyrrole, furfuran, and their derivatives. The reagent we used was that of Carr and Price. Antimony trichloride was washed several times with chloroform and dried in a desiccator. A saturated solution of this product was made by adding 30 gm. to 100 cc. of chloroform (U.S.P.), allowing it to stand, and decanting the clear liquid. The chloroform contained 1 per cent alcohol. The compounds to be tested were made up to 20 per cent chloroform solutions or, if insoluble, into 20 per cent chloroform suspensions. To the 3 drops of the chloroform solution or suspension were added 2 cc. of the antimony trichloride solution. The characteristic color may not form immediately, but develops on standing.

We have modified the above procedure by adding 0.5 cc. of acetic anhydride to the substance dissolved in chloroform subsequent to admixture with the antimony trichloride reagent. The acetic anhydride serves as a chromogenic stimulator. For the same purpose Whitby (29) and Rosenheim (30) have used formaldehyde. Brode and Magill (31) have employed acetic anhydride with their antimony trichloride reagent in their experiments on fish liver oil. As the reason for their use of the anhydride they state that it serves to remove hydrochloric acid and water from the reaction mixture. To illustrate the chromogenic power of acetic anhydride, we will take skatole as an example. This compound gives with antimony trichloride in the presence of acetic

anhydride a light purple color developing in 10 minutes to a very deep royal purple. Skatole with antimony trichloride alone gives a yellow color with a slight tinge of red only after 24 hours.

Besides acting in the capacity of a substance capable of stimulating color production, acetic anhydride acts as a solvent by its ability to form acetyl derivatives. Thus pyrrole fails to give color with antimony trichloride because it forms a white gelatinous precipitate. When, however, acetic anhydride is added before the antimony trichloride solution, precipitation is prevented and a characteristic color is permitted to form. The mixture without acetic anhydride we have designated as Reaction Mixture A and the one with acetic anhydride as Reaction Mixture B. We have always found that mixtures with acetic anhydride gave a clearer and more distinct color than mixtures without the anhydride.

*Pyrrole*—Compounds of the pyrrole type yield an intense color with Reaction Mixture B. The only exceptions are proline, which is a tetrahydropyrrole with a carboxyl group, and nicotine, which is composed of a pyridine nucleus and a reduced pyrrole, and nicotine salicylate. Both nicotine and its salicylate form precipitates in the reaction mixtures with or without acetic anhydride (Table I).

Hemoglobin, hematin, and the bile pigments, bilirubin and biliverdin, each contain four pyrrole rings. Hemoglobin gives no reaction. It is insoluble in chloroform. Hematin, bilirubin, and biliverdin dissolve in chloroform and yield a characteristic color with antimony trichloride in the presence of acetic anhydride. Since the chloroform solutions of these three pigments are very deep in color, it is imperative to make very dilute solutions in order to recognize the particular color obtained as a result of the interaction with antimony trichloride. Chlorophyll gives no reaction with antimony trichloride, but with acetic anhydride and the antimony compound, a yellow color with a tinge of green develops after prolonged standing in a solution of the plant pigment so dilute as to produce an almost colorless liquid.

We have tested a number of heterocycles which do not belong to the 5-membered monoheterocyclic series. Among them may be mentioned histidine, a pentacycle with 2 nitrogen atoms in the ring, and the monoheterocyclic 6-membered compounds, quinoline, quinolinic acid, quinaldinic acid, and cinchophen. These substances give no reactions. Acridine, however, displays

TABLE I

*Antimony Trichloride Reactions with Compounds Containing 5-Membered Monoheterocyclic Rings. Pyrrole*

Type and name of compound	Reaction mixture	Result
Pyrrole	A	Gelatinous gray-white ppt.
	B	Brick-red or cherry-red; intensity increases on standing
<i>n</i> -Methylpyrrole	A	Chalky white ppt. on addition of acid; lemon ppt. at bottom of tube
	B	Lemon, changing to light yellow-orange then to dark brick-red
<i>n</i> -Ethylpyrrole	A	White chalky turbulence, yielding gradually brown viscid ppt.; on standing liquid becomes colorless, ppt. red-brown
	B	Lemon-yellow, changing to orange, gradually turning deep orange or red
<i>n</i> -Butylpyrrole	A	White chalky turbulence at first, finally lemon substance settling at bottom of tube
	B	Brown-yellow, changing to yellow-orange, gradually darkening, finally becoming deep cherry- or brick-red.
<i>n</i> -Phenylpyrrole	A	Lemon-yellow turbidity, finally red-brown viscous liquid separating at bottom
	B	Lemon, changing to deep orange or blood-orange
<i>n</i> - <i>o</i> -Tolylpyrrole	A	Lemon tinted turbidity, gradually changing to clear lemon liquid and dark brown ppt.
	B	Lemon, changing to yellow-orange, finally to blood-orange
<i>n</i> - <i>m</i> -Tolylpyrrole	A	Clear liquid with deposit of brick-red substance at bottom of tube
	B	Brick tint immediately changing to lemon while pouring in antimony trichloride solution, changing to light yellow-orange, orange to brick-red, finally to wine
Proline (pyrrolidine-carboxylic acid)	A	No reaction; slight turbidity
	B	" "
Indole-3- <i>n</i> -propionic acid (benzopyrrole-3- <i>n</i> -propionic acid)	A	Does not go into solution; straw-colored liquid
	B	Reddish brown, changing to deep wine, finally to brownish red

TABLE I—*Concluded*

Type and name of compound	Reaction mixture	Result
Skatole (methylinole)	A	Yellow with tinge of brown, giving after few days cherry-red or carmine-red
	B	Light purple developing in 10 min. to very deep royal purple
Tryptophane ( $\beta$ -indole- $\alpha$ -aminopropionic acid)	A	No change at first; on long standing deep carmine-red dense liquid separates at bottom
	B	Deep lemon or yellow-brown
Carbazole (dibenzo-pyrrole)	A	Does not go into solution in reaction mixture; on long standing suspended particles turn bluish or greenish blue
	B	Light reddish brown; changing to wine, finally to purple
Bilirubin (4 pyrrole rings)	A	Deep orange liquid; from dilute yellow solutions; on standing colorless liquid and red-brown ppt.; after few days ppt. turns bluish green and solution assumes bluish tinge
	B	Light orange liquid; on standing becomes yellow-brown; after few days becomes green
Biliverdin (4 pyrrole rings)	A	Greenish ppt.
	B	On long standing dilute reaction mixtures become dark green
Hemoglobin (4 pyrrole rings)	A	Insoluble in reagents used; no reaction
Hematin (4 pyrrole rings)	B	" " " " " "
	A	On standing brown ppt. and clear colorless liquid
Chlorophyll (4 pyrrole rings)	B	On standing gradually becomes dark brownish red
	A	No reaction
Nicotine, nicotine salicylate (1 pyrrole ring)*	B	On long standing solution turns yellow with greenish tinge
	A	White ppt. formed
	B	" " "

\* The ring is reduced pyrrole.

with antimony trichloride green fluorescence, and with the same reagent and acetic anhydride the fluorescence is somewhat more intense.

*Thiophene*—Thiophene is the only sulfur-containing hetero-

cycle we have studied. With the antimony trichloride reagent the color develops but very slowly, finally yielding a brownish red. With the reagent modified by the addition of acetic anhydride the color reaction is more rapid and more intense, and a purplish blue color develops changing to light blue liquid and forming a dark blue precipitate. The color of the liquid and of the solid remains unchanged for many weeks. We are attempting to prepare selenophene in order to test out its behavior with reference to the antimony trichloride reagents.

*Furan (Furfuran)*—We have extended our experiments with the antimony trichloride reagents to include furfuran and its derivatives.<sup>1</sup> We have found compounds of the furan or furfuran type to react very strongly, very often with the formation of a blue color. Furfuran itself yields a green color with antimony trichloride, changing to greenish blue and finally to a blue liquid with a dark brown precipitate. With the modified reagent with acetic anhydride a deep purple wine color is obtained changing to purple and finally to blue. The color reaction for each particular furfuran derivative is to be found in Table II. With many of the compounds that give a greenish or bluish liquid a purplish or bluish precipitate formed after prolonged standing.

The intensity and the variety of color depend on the presence of the particular substituent in the furan ring. As a rule the presence of the aldehyde group strengthens the color and its permanence. Derivatives of furfuran aldehyde (furfural) such as the oxime and the condensation products, such as furoin, furil, furfural acetone, furlacrolein, furlacrolein oxime, furfural acetophenone, and furfural diacetate, react even more promptly and more intensely than the mother substance.

The presence of the hydroxyl group slows up the reaction and reduces its intensity. The presence of a carboxyl group attached to the heterocycle, be it pyrrole or furfuran, inhibits the reaction altogether. Proline, which is pyrrolidenecarboxylic acid, gives

<sup>1</sup> We are indebted to the Quaker Oats Company for the following compounds: furfural, furfural acetone, furfuryl alcohol, tetrahydrofurfuryl alcohol, hydrofuramide, the methyl, ethyl, propyl, and amyl esters of furoic acid and sodium furacrylate. Chlorophyll was secured from Eimer and Amend. All the other compounds employed were obtained from the Eastman Kodak Company.

TABLE II

*Antimony Trichloride Reactions with Compounds Containing 5-Membered Monoheterocyclic Rings. Thiophene and Furfuran*

Type and name of compound	Reaction mixture	Result
Heterocycles with sulfur atom		
Thiophene	A	Colorless at first, finally changing after few days to light straw-color, then to brownish red
	B	Colorless at first, later developing pinkish purple, finally changing to blue and blue ppt.
Heterocycles with oxygen atom		
Furfuran (furan)	A	Green, greenish blue, blue, and dark brown ppt.; liquid becomes yellowish green overnight
	B	Deep purplish wine, changes to purple in 20 min., 5 min. later to purplish blue, finally to blue
Furfuryl alcohol	A	Dirty lemon ppt., purplish liquid changing to blue, finally to purple
	B	Dark brown-red changing to purplish, greenish blue, and blue
“ acetate	A	Brown liquid and dark brown ppt. with oily drops at bottom of tube; azure blue ppt. on bottom, straw-colored liquid above; on standing overnight, deep purple-brown ppt. on sides of tube, finally becoming deep royal purple
	B	Instantaneous dark blue
Tetrahydrofurfuryl alcohol	A	Deep greenish blue suspension
	B	On standing deep royal purple
Tetrahydrofurfuryl acetate	A	Light lemon rapidly changing to orange-brown and more slowly to purplish brown, to reddish brown
	B	Light lemon to darker lemon, green, amber, brownish green, dark green
Tetrahydrofurfuryl butyrate	A	Straw color, golden brown, green, purple with light brown tinge, light purple, darker purple
	B	Lemon, reddish lemon, pink-red, dark reddish purple



TABLE II—Continued

Type and name of compound	Reaction mixture	Result
Tetrahydrofurfuryl lactate	A	Yellow straw color, golden brown; red-brown overnight
	B	Yellow straw color, golden brown; brown overnight
Furfural	A	Greenish brown, blue, purplish blue
	B	" " " "
Furoin	A	Light brown ppt.; liquid green
	B	Clear green liquid
Furil	A	Brownish yellow and darker ppt., yellowish green liquid
	B	Brownish yellow changing to yellowish green
Furfuraldoxime	A	White ppt., on standing thick brownish liquid forms on bottom
	B	Lemon, cherry-red, wine, reddish purple, deep purple
Furfural acetone	A	Dark orange, turning deep red with brown tinge
	B	Green, becoming darker on standing
Furylacrolein	A	Amber somewhat turbid; overnight yellowish green liquid and greenish brown on sides of tube
	B	Lemon-green, wine, dark red, blue specks on side of tube, bluish green; on standing blue liquid and blue ppt.
" oxime	A	Lemon-green solution with yellowish brown ppt. on standing
	B	Green-yellow, changing to green, to red-brown, to very dark green, finally to purple
Furfural acetophenone	A	Lemon-yellow changing to brown
	B	Lemon, finally developing into deep lemon-green or olive-green
" diacetate	A	On standing light bluish purple
	B	Lemon, reddish lemon, pink-red, dark reddish purple
Hydrofuramide	A	Greenish muddy liquid, and dark blue-green ppt. on bottom and sides of tube
	B	Reddish brown, brown with greenish blue tinge, becoming deep bluish green on standing

TABLE II—*Concluded*

Type and name of compound	Reaction mixture	Result
Furonitrile, furoamide, furoyl chloride, furoic acid	A	No reaction
	B	" "
Methyl, ethyl, propyl, butyl, amyl, and iso-amyl esters of furoic acid	A	" "
	B	" "
Furacrylic acid	A	Yellow liquid with slight greenish ppt. on standing
	B	On standing very light lemon with brown tinge
Furylacrolyl chloride	A	On standing overnight light brownish green liquid and greenish blue ppt.
	B	On standing very light lemon with brown tinge
Furylacryl amide	A	Colorless liquid, blue ppt. on bottom and sides of tube
	B	On standing light lemon, finally assuming green tinge
Strophanthin (isostrophanthidin contains 1 furan ring)	A	Colorless liquid and flaky translucent solid turning to creamy and gradually darker brown. After few days greenish blue on sides and bottom of tube
	B	Colorless at first, gradually develops a brownish amber, finally olive-green

no reaction. Furoic acid does not react; nor do any of its derivatives which involve substitution in the carboxyl group. Furoyl chloride, furoamide, and furonitrile and esters of furoic acid are therefore unresponsive. The fact that oxidation of the aldehyde prevents the reaction, coupled with the findings that vitamin A when subject to oxidation is no longer potent biologically and no longer gives the characteristic color reaction, leads to the belief that vitamin A is either an aldehyde or an alcohol. Cady and Luck (32), working from another angle, came to the conclusion that vitamin A is probably an aldehyde, while Karrer, Morf, and Schöpp (33) consider vitamin A to be an alcohol.

When, however, the carboxyl group is on the side chain and not directly attached to the ring, as in the case of furacrylic acid, the

reaction, although positive, is not intense and develops but very slowly. Furylacryloyl chloride and furylacrylamide behave like furacrylic acid. Tryptophane is  $\beta$ -benzopyrrole or  $\beta$ -indole- $\alpha$ -aminopropionic acid. The carboxyl group is on the side chain. The reaction is weak, yielding with antimony trichloride and acetic anhydride an atypical color, deep yellow or yellow-brown. This atypical yellow color has also been reported by Heilbron and Spring (23) for  $\alpha$ - and  $\beta$ -isoergosterol and for dihydroergosterol. Hemoglobin, bilirubin, biliverdin, and chlorophyll react very feebly. These structurally complex compounds possess carboxyl groups.

Degree of unsaturation is held responsible for the antimony trichloride color reaction with unsaturated compounds, according to von Euler and Hellström (34). We have found that complete saturation of the furfuryl radical does not abolish color formation. Tetrahydrofurfuryl alcohol yields a deep royal purple with antimony trichloride and a deep greenish blue liquid and precipitate with the same reagent in the presence of acetic anhydride. Tetrahydrofurfuryl acetate, butyrate, and lactate also yield characteristic color reactions. Proline is a saturated pyrrole to which is attached a carboxyl group. It is negative in its behavior towards antimony trichloride because of the interference of the carboxyl group.

Strophanthin gives with Reaction Mixture A a colorless liquid with flaky gray-white solid particles which gradually turn greenish blue. This result is interesting in view of the fact that Jacobs and Gustus (35) and Jacobs and Elderfield (36) indicate the presence in strophanthidin and in isostrophanthidin of two 6-membered cyclic rings and one 5-membered heterocyclic ring, which is a furfuran ring.

The 6-membered monoheterocyclic compounds containing an oxygen atom, xanthone, and xanthidol, do not react with the antimony trichloride reagent.

We have included chaulmoogra oil in our tests because it contains two compounds, chaulmoogric acid and hydnocarpic acid, the structures of which indicate the presence of a pentacyclic ring. This ring, which is isocyclic and not heterocyclic, is found in cholesterol (37), in ergosterol (23), and in the bile acids (38). The two samples of the chaulmoogra oil tested gave somewhat different results (Table III). We have also examined a sample of chaul-

TABLE III  
*Antimony Trichloride Reactions with Various Oils*

Name of oil	Reaction mixture	Result
Almond oil, sweet	A	Straw color changing to pink, to deep golden yellow; brown color developing overnight with yellowish ppt.
	B	Almost colorless, with faint straw color, changing to pink; deep brown with purple tinge developing overnight
Castor oil	A	Cloudy, straw color with pink tint; yellow ppt. overnight and golden yellow color similar to that of bilirubin
	B	Almost colorless liquid with pinkish tint; dark brown with tint of pink developing overnight
Cottonseed oil	A	Brownish gold, developing into deep orange, finally cherry-red and chocolate-brown ppt.
	B	Straw color, developing into brown with purple tinge, finally purplish wine
Linseed oil	A	Cloudy gold to cloudy wine; brownish wine with grayish ppt. developing overnight
	B	Amber, grayish amber, pinkish brown; brownish wine developing overnight
Olive oil	A	Straw color developing into cloudy light orange; gray ppt. developing overnight and light brown color
	B	Light amber, developing overnight light tan, finally changing to pinkish brown
Sesame oil	A	Golden yellow changing very soon to cherry-red, to wine, to mahogany-brown; developing overnight to chocolate-colored ppt.
	B	Light amber, pinkish yellow, light brown, purplish brown; developing overnight to purplish brown
Wheat germ oil	A	Bluish green; overnight cloudy brown
	B	" " " "
Chaulmoogra oil (Eli Lilly and Company)	A	Slight lemon turbid mixture
	B	Clear slightly lemon liquid, turning to light amber
Chaulmoogra oil (Sargent and Company)	A	Olive color, changing to yellow
	B	Lemon-olive changing to brown with greenish tint, finally reddish brown

TABLE III—*Concluded*

Name of oil	Reaction mixture	Result
Chaulmoogric acid	A	Lemon-yellow cloudy turbidity, finally turning to olive
	B	Clear lemon-yellow liquid, changing to brownish yellow, finally to brown
Ethyl chaulmoograte	A	Yellowish olive turning yellow
	B	" " " to greenish olive, finally to purplish red
Chaulmestrol	A	Brown changing to mahogany tint
	B	" with greenish tint, changing to dark greenish olive, finally to purple

moogric acid and a sample of ethyl chaulmoograte. Both came from the Eastman Kodak Company and were marked "practical." A sample of chaulmestrol, a trade name for the ethyl esters prepared from chaulmoogra oil, gave with antimony trichloride in the presence of acetic anhydride, a brown color with a greenish tinge, which changed to dark greenish blue and finally to purple.

*Antimony Trichloride Reaction with Oils Other Than Liver Oils*—Harden and Robison (39) maintain that the purple color obtained by the addition of sulfuric acid to cod liver oil bears a resemblance to Neuberg and Rauchwerger's test (40) for cholesterol in which the reagent is  $\psi$ -methyl furfuraldehyde obtained from rhamnose by means of concentrated sulfuric acid. Harden and Robison postulated the presence of a furfuran derivative in fish oil, since they were able to obtain a similar color reaction by the interaction of sulfuric acid with furfural and cholesterol dissolved in petroleum or in chloroform. They were, however, unable to isolate from coalfish oil by distillation with steam or under reduced pressure or by other methods either furfural or some compound which could replace it in the reaction system, cholesterol-furfural-sulfuric acid.

The presence or absence of furan derivatives in oils needs, nevertheless, further investigation. Shear (41) reported a color reaction with cod liver oil obtained by the use of a reagent consisting of 15 volumes of aniline to 1 volume of concentrated hydrochloric acid. This reagent yields a characteristic color reaction with furfuran and its derivatives, with ionone and carotene (42), and with sterols (43). In this connection it is of great interest to men-

tion that Tocher (44) isolated from sesamine oil a crystalline, unsaponifiable compound, and named it sesamine. Bertram, van der Steur, and Waterman (45) recently demonstrated a furfuran ring in its molecule.

We have tested sesame oil with the antimony trichloride reagents. The oil reacted very strongly with antimony trichloride with the development of a golden yellow color which rapidly transformed to a cherry-red or wine-red color. In the presence of acetic anhydride sesame oil finally developed a clear, deep purplish color.

We have also tested olive oil, cottonseed oil, and wheat germ oil. Precipitates formed in all the reaction mixtures containing antimony trichloride, but not in those containing the antimony salt with acetic anhydride. Norris and Church (7) also examined thirty-four varieties of essential oils in chloroform solution with antimony trichloride. They reported that three did not react at all. Others gave various shades of yellow, brown, and red. Oil of wormwood gave a green color, ethereal oil gave a purple color, and cedar wood oil gave an intense blue, which showed an absorption band with a maximum at  $580\text{ m}\mu$ . They attribute the reactivity of the essential oils to the presence of unsaturated compounds.

Wheat germ oil developed a cloudy bluish green with antimony trichloride and a clear bluish green coloration in the reaction mixture containing acetic anhydride. Willimott and Wokes (4) reported a positive test when antimony trichloride reacted with decolorized yellow maize oil. Croxford (46) and Stout and Schuette (47) also obtained a positive test with rye germ oil.

In order to determine the effect of oxidation on the color reaction, we heated a sample of wheat germ oil at  $100^\circ$  for 1 hour while passing oxygen through it. At the end of that time the reaction was still positive and even stronger. We expected to find the reaction negative in view of the destructive action of oxygen on vitamin A. Hopkins (48), Drummond and Coward (49), and Zilva (50) reported loss of vitamin A potency at high temperatures in the presence of oxygen or an oxidizing agent, although Sherman and his coworkers (51) and Cady and Luck (32) reported that the stability of this vitamin was greater in plant than in animal substance, and although Dann (52) observed that the vitamin may resist the action of oxygen, the solvent and impurities therein reacting as inhibitors.

Increase in the intensity of the antimony trichloride reaction has also been noted by other investigators. Hawk (13) observed a more intense reaction after exposing cod liver oil to air and sunlight for a total of 79 hours. Mittlemann (53) autoclaved at 120° cod livers sealed in air-evacuated tin cans and observed that the liberated oil gave no blue color with antimony trichloride immediately on opening after cooling. The same oils, however, examined 10 days later gave a strongly positive test. Lovern, Creed, and Morton (54) observed that codling liver oil exposed to the air at 70° or oxygenated at the same temperature underwent a considerable augmentation in its capacity to give the blue color with antimony trichloride. Heilbron, Gillam, and Morton (55) maintain that liver oils contain two different substances each capable of reacting with antimony trichloride and each capable of yielding in the reaction mixture a characteristic absorption spectrum. One compound on treatment with ozonized air, hydrogen peroxide, or benzoyl peroxide yields with antimony trichloride a deeper color reaction and develops at the same time an increase in the intensity of the band at 606  $m\mu$ . Under similar treatment with antimony trichloride the other compound does not seem to show intensification of the color reaction, nor does the reaction mixture show alteration in the band at 527  $m\mu$ .

The existence of two chromogenic substances in liver oils has also been postulated by Morton, Heilbron, and Thompson (56), Emmerie, van Eekelen, and Wolff (57), Morton (12), and Ender (58). One of these chromogenic compounds is vitamin A, which is easily oxidized; the other is a compound not destroyed by oxygen or oxidizing agents. Our results with wheat germ oil indicate the presence of a chromogenic substance not affected by oxygen or oxidizing agents.

*Relation of Vitamin A to Substances Reacting with Antimony Trichloride*—Antimony trichloride gives characteristic reactions with oils containing vitamin A, with many sterols and their derivatives, with the carotenoid pigments, and with compounds containing 5-membered monoheterocyclic rings. It would be of interest to determine whether sterols, carotenoid pigments, and compounds containing 5-membered monoheterocyclic groupings have the ability to develop vitamin A potency. Kerppola (59) prepared from cod liver oil a cholesterol fraction differing from ordinary

cholesterol, which acts as a chromogen towards antimony trichloride, cures xerophthalmia, and restores reactivity with antimony trichloride to tissues of vitamin A-depleted rats. Seel (24) and Seel and Dannmeyer (60) oxidized cholesterol with benzoyl peroxide, according to the method of Lifschütz, and obtained a product, presumably oxycholesterol, resembling vitamin A in giving ultra-violet absorption bands at  $327\text{ m}\mu$  and  $293\text{ m}\mu$ , in yielding the typical color reaction with antimony trichloride, and in curing xerophthalmia. The minimum daily dose of the oxidized cholesterol required to cure xerophthalmia was 0.1 mg., whereas a natural vitamin preparation from shark liver oil proved active in 0.0001 mg. doses. Seel expressed the view that vitamin A is a very highly labile partial oxidation product of cholesterol.

That carotene possesses vitamin A potency has been definitely established by a number of workers (18, 61-67). Both  $\alpha$ - and  $\beta$ -carotene (68) possess biologic potency; so do also  $\alpha$ - and  $\beta$ -dihydrocarotene (69). Chlorophyll carries in its molecule four groupings of the 5-membered heterocycle, pyrrole. Burgi (70) has demonstrated in rats receiving no vitamin A that growth was started by the administration of chlorophyll and its derivatives phacophytin and chlorophyllin. The pigments were free from carotene. In this connection we may state that Abbot (71) has reported that crystalline chlorophyll does not cure xerophthalmia. Burgi (72) has published work indicating that rhodin *g*, obtained from chlorophyll *b*, also stimulates growth in the absence of vitamin A in the diet.

#### SUMMARY

Antimony trichloride in chloroform solution gives characteristic color reactions with the 5-membered monoheterocyclic compounds, pyrrole, thiophene, and furfuran, and with more complex compounds containing pyrrole or furfuran configurations.

The color reaction is intensified and often modified by the addition of acetic anhydride. The anhydride catalyzes the chromogenic activities of the heterocycles. It serves to remove water and free hydrochloric acid from the reaction mixture. It also aids in holding the heterocyclic compounds (the pyrroles) in solution.

Derivatives of the monoheterocyclic compounds also yield characteristic colors, the substituents in the molecule modifying the



intensity and rapidity of the reaction. As a rule the presence of the hydroxyl group shows up the reaction and reduces its intensity. The aldehyde reacts more vigorously than the corresponding alcohol. The carboxyl group attached directly to the heterocycle, as in the case of proline and furoic acid, inhibits the reaction altogether. When, however, the carboxyl group is on a side chain and not attached directly to the ring, as in the case of tryptophane and furacrylic acid, the reaction though positive is not intense and develops but very slowly. The acyl chloride, the amide, and the nitrile behave like the corresponding acid obtained on hydrolysis.

The color reaction obtained with antimony trichloride in chloroform solution is not specific for vitamin A, since positive tests have been obtained with sterols, with pigments other than carotene, and with such 5-membered monoheterocyclic compounds as thiophene, furfuran, and many of its derivatives. The color reaction may, however, prove to be indicative of the presence of a specific grouping or configuration in the vitamin A molecule.

The fact that a group of chemical compounds besides the sterols and carotenoids reacts with antimony trichloride lends support to the idea that fish liver oils contain beside vitamin A another compound possessing chromogenic properties.

Compounds other than vitamin A possessing chromogenic properties with reference to antimony trichloride are in all probability common components of animal oils rich in vitamin A as well as of vegetable oils devoid of vitamin A.

The chromogenic compound not possessing vitamin A potency gives when oxygenated or when treated by oxidizing agents a more intense antimony trichloride reaction.

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## THE NATURE OF THE ALLEGED MOLECULAR SIEVE MEMBRANES

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One of the most outstanding electrical properties of tissues is the great variability of many biological potential differences with the concentration of solutions in contact with the tissues. This variability nearly equals that of metallic electrodes (1). A certain resemblance of tissue membranes to metallic electrodes is thus indicated. This resemblance may, in time, lead us to a better understanding of the nature of the electrical polarization in tissues, which somehow bears a relation to the origin of excitability ((2) p. 250 ff.). Every attempt ought to be made, therefore, to understand the physical nature of this important electrical property of tissues.

This electrode-like action of membranes can be explained as a result of non-proportional distribution of salts due to a phase boundary potential, as was shown 20 years ago by one of us (Beutner (2, 3)). The proportionality of salt distribution for which the well known law of partition provides is disturbed by the intervention of a chemical reaction which occurs at the phase boundary. This explanation was verified by experiments on salt distribution and other independent experimental evidences (2, 3).

In 1925, Michaelis and coworkers (4-15)<sup>1</sup> observed that well dried collodion films also show this electrode-like electromotive action, and propounded an entirely different theory; viz., that these forces come about not through phase boundary potentials but by dint of electrically charged pores which act as ionic filters. Mi-

<sup>1</sup> Whereas from the statements of Michaelis the theory appears hardly more than a hypothesis, Höber and his pupils, notably Mond and coworkers, seem to consider it as well established (16, 17).

Michaelis admits that this explanation is not of necessity to be applied to the electrode-like action of plant cuticula as observed by Loeb and Beutner in 1911 (1). Even his experiments demonstrating the inhibition of diffusion in the absence of a salt furnish no evidence for this theory as he himself admits. For, it is readily understood that a chemical reaction may produce the same effects: the potassium salts in the apple juice, reacting with some unknown substance in the cuticula, allow the potassium to enter into the cuticula; this reaction product then in turn reacts with the outside NaCl solution to form potassium salt in the outside aqueous solution; however, if no salt is present in the outside water no reaction and hence no diffusion occurs as the experiments show to be the case.

Nevertheless, Michaelis denies the applicability of this simple theory to explain the electrode-like action of a dried collodion film for reasons which are best quoted in his own words (18).

"Completely dried collodion is one of the very best electric insulators. When touched with wool or hair it becomes strongly electrified and will retain this charge over a long period. When such a membrane is thick enough and separates two electrolyte solutions, the conductivity is, at the beginning, almost zero. It increases, however, rapidly with the progress of imbibition by the membrane. Now it would be absurd to suggest that water or a potassium chloride solution could go into 'solid solution' in the dried collodion within some minutes. . ."

It is difficult to see why it should be absurd to suggest that water and salt go into solid solution in nitrocellulose, at least in the surface layer. The thorough investigations of Northrop (19) have shown that gases and vapors diffuse through a dry collodion membrane *by dissolving in the substance of the membrane. They do not pass through holes in the membrane* since the rate of diffusion would then be inversely proportional to the square root of the density, which is not the case (Northrop (19)). Hence, water also must dissolve in the substance of collodion, which would account for the increase of conductivity even if the filling of the pores plays no part. Numerous other experiments of Northrop (19), all of which cannot be reviewed here, favor the assumption of solution in the collodion rather than of a passage through the pores.

Moreover, the possibility of chemical reactions at the surface of

the collodion must be considered.<sup>2</sup> Since collodion, or nitrocellulose, is a highly reactive substance, it seems likely that it is capable of entering into surface reactions even with aqueous solutions of KCl or NaCl whereby HCl and a sodium compound of nitrocellulose are formed, the latter being held fast by the nitrocellulose itself, while HCl should diffuse into the water. Hence, if collodion is shaken with an aqueous KCl (or NaCl) solution, free HCl detectable by titration should appear in the solution.

Titration experiments have shown that in this case HCl can actually be detected. We have to consider in this connection that collodion will give off traces of acid even when shaken with pure water—probably due to impurities contained in it—but, when collodion is shaken with a salt solution, much more acid is formed. According to our theory such a chemical reaction between collodion and NaCl should be the cause of the effect of concentration of the collodion film, just as it is the case for other substances which show the effect of concentration, as explained before (4-17).<sup>1</sup>

The experiments which demonstrate this formation of acid were performed as follows: Celloidin (Schering), which is a semisolid mass containing varying amounts of ether and alcohol was used; cubes of equal size of this material were cut. An equal number of these were placed in equal volumes either of 0.1 M KCl solutions or of distilled water. Both lots were shaken simultaneously for 48 hours. Equal amounts of the aqueous fluids were then pipetted into Erlenmeyer flasks and titrated with 0.01 N NaOH to identical pH values, with phenolphthalein as indicator.

*Experiment 1 (by R. B.). 64 Cubes in Each 50 Cc. of Water or of 0.1 M KCl—30 cc. were titrated.*

0 75 cc	0 01 N NaOH used for	0 1 M KCl
0 675 "	0 01 " " " "	water

*Experiment 2 (by R. B.). 100 Cubes in Each 30 Cc. of Water or of 0.1 M KCl—20 cc. were titrated.*

2 1 cc.	0 01 N NaOH used for	0 1 M KCl
1 6 " "	0 01 " " " "	water

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<sup>2</sup> Numerous solid substances are known to react superficially with a solution in contact with them, *e g.* permutit. For further examples see Beutner ((2) pp. 139, 140).

*Experiment 3 (by R. B.). 100 Cubes in Each 25 Cc. of Water or of 0.1 M KCl—20 cc. were titrated.*

2.1 cc. 0.01 N NaOH used for 0.1 M KCl

1.6 " 0.01 " " " " water

*Experiment 4 (by R. B.). 100 Cubes in Each 25 Cc. of Water or of 0.1 M KCl—20 cc. were titrated.*

2.4 cc. 0.01 N NaOH used for 0.1 M KCl

1.65 " 0.01 " " " " water

The KCl used in these experiments was titrated and found to be absolutely free from perceptible traces of acid.

In the following experiments, which were performed with other salts, the possibility of a salt error was excluded in a still more rigid fashion. For this end, after shaking pure water with the celloidin cubes, the same salt was added to this water in the same amount as in the respective salt solution which had been shaken with the celloidin cubes simultaneously. In other words, *the two aqueous fluids which were titrated contained in every instance the same amount of the same salt, a difference existing between them merely in that in the one case this salt was added before, in the other case after the shaking with the celloidin cubes.* Hence in the former case, the salt was capable of reacting with the celloidin cubes; in the latter case, no reaction was possible. A salt error is thus manifestly obviated. The titrations indicate that much more acid was present in those cases in which the dissolved salt had come into contact and reacted with the celloidin. Such experiments were performed, *e.g.* with NaCl solution, yielding the following results.

*Experiment 5 (by M. C.). 100 Cubes Shaken with Each 30 Cc. of Water or of 0.1 M NaCl—20 cc. were titrated.*

2.86 cc. 0.01 N NaOH used for 0.1 M NaCl

0.98 " 0.01 " " " " water

Another experiment was performed (by W. M. L.) with a new batch of collodion, the shaking being continued in this case for more than a week. The results agreed with the previous ones as closely as one might reasonably expect, considering that the surfaces of such cubes cannot be made absolutely alike in two subsequent experiments.

*Experiment 6 (by W. M. L.). 93 Celloidin Cubes in Each 25 Cc. of Water or of 0.1 M NaCl—20 cc. were titrated.*

3 01 cc. 0 01 N NaOH used for 0 1 M NaCl  
1 29 " 0 01 " " " " water

Analogous experiments were performed with other salts, viz. with *lithium chloride* and *calcium chloride*, yielding the following results.

*Experiment 7 (by W. M. L.). 30 Celloidin Cubes in Each 25 Cc. of Water or of 0.1 M CaCl<sub>2</sub>—10 cc. were titrated.*

0 19 cc. 0 01 N NaOH used for 0 1 M CaCl<sub>2</sub>  
0 07 " 0 01 " " " " water

Hence for 20 cc.

0 38 cc. 0 01 N NaOH used for 0 1 M CaCl<sub>2</sub>  
0 14 " 0 01 " " " " water

*Experiment 8 (by W. M. L.). 30 Celloidin Cubes in Each 25 Cc. of Water or of 0.1 M LiCl—10 cc. were titrated.*

0 37 cc. 0 01 N NaOH used for 0 1 M LiCl  
0 07 " 0 01 " " " " water

Hence for 20 cc.

0 74 cc 0 01 N NaOH used for 0 1 M LiCl  
0 14 " 0 01 " " " " water

For LiCl and CaCl<sub>2</sub> this differential effect appears to be even larger than for KCl and NaCl, primarily due to the low titration value in the "water" control (compare the titration figures of water shaken with collodion cubes). These low figures are due to the hydrolytic splitting of the LiCl or CaCl<sub>2</sub> added after the shaking. Both the LiCl and CaCl<sub>2</sub> used were not chemically pure, but were really basic salts; consequently, our LiCl and CaCl<sub>2</sub> solutions turn pink to phenolphthalein after the addition of less alkali than do KCl and NaCl solutions. In fact, we found that in order to render 20 cc. of 0.1 M KCl or 0.1 M NaCl distinctly pink to phenolphthalein, as much as 0.1 cc. of 0.01 M NaOH must be added, while for the LiCl and CaCl<sub>2</sub> solutions no more than 0.02 cc. was needed.

The experimental findings with CaCl<sub>2</sub> and LiCl solutions would indicate, according to our theory, that these salts show an effect of concentration in the same manner as do sodium and potassium



salts. According to Michaelis and Fujita (6) this is the case although the effect for  $\text{CaCl}_2$  is described by them to be much smaller and very variable. We have convinced ourselves of this fact by repeating the electric measurements described by Michaelis; the electromotive effects of concentration produced if a well dried collodion film was in contact with  $\text{CaCl}_2$  we found were less than one-half of that observed if  $\text{NaCl}$  or  $\text{KCl}$  was used,  $\text{LiCl}$  occupying an intermediate position. Nevertheless, we have found that a considerable amount of acid is formed if  $\text{CaCl}_2$  solutions are shaken with collodion, as stated. This is not in disagreement with our theory since the diminished electric effect of  $\text{CaCl}_2$  need not be due to a less intensive chemical reaction, but is probably *the result of an interference of the acid formed with the effect produced by  $\text{CaCl}_2$*  for the following reasons. The effect of dilution consists in a shifting of the potential difference toward the positive side;  $\text{CaCl}_2$ , however, gives a markedly positive potential even at high concentrations. According to Michaelis' figures (which we have verified)<sup>3</sup> 0.1 M  $\text{CaCl}_2$  is more than 100 millivolts positive relative to 0.1 M  $\text{KCl}$  and more than 150 millivolts positive relative to  $\text{HCl}$ . It is difficult, therefore, to shift the potential difference further to the positive side by diluting the  $\text{CaCl}_2$ . The potential difference is markedly influenced in this case by the slightest amounts of acid present. Since acid is formed at the phase boundary, and since, moreover, such slight amounts may be present *a priori* in the diluting water owing to  $\text{CO}_2$  dissolved and other conditions, it is evident that a dilution of a  $\text{CaCl}_2$  solution can only yield indefinite results.

Such a marked influence of the very lowest acid concentrations must also be expected according to the experiments on phase boundary potentials formerly developed by one of us ((3) p. 29).

We have convinced ourselves of the correctness of this relationship through the following experiments.

<sup>3</sup> For this end we prepared a thoroughly dried collodion film of the kind described by Michaelis as the flat type. A glass tube about 0.5 cm. wide was immersed with one end in a celloidin solution and the film thus formed allowed to dry up, so as to form a closed bottom over one end of the glass tubing. 0.1 M  $\text{KCl}$  was poured in the tube, and the tube itself was immersed in a solution of  $\text{CaCl}_2$  solution contained in a beaker of 50 cc. content.

The electromotive force of the system (in one case)



was measured and found to be 0.038 volt, the positive pole being at the  $\text{CaCl}_2$  side. A trace of HCl solution was then added, *viz* so little of it that the  $\text{CaCl}_2$  remained practically unaltered, but a HCl concentration of 0.00001 M was now present. *This diminutive amount of acid threw off the electromotive force entirely, the polarity of the system became reversed*, and the electromotive force was now 0.041 volt, hence the total change was  $0.038 + 0.041 = 0.079$  volt. Upon repeating the experiment an even larger variation, *viz* 0.135 volt, was observed. In still another case upon addition of 0.0001 M HCl a change of 0.11 volt was seen. (It should be remembered that the potential differences of the collodion film in contact with  $\text{CaCl}_2$  solutions are variable in every case.)

Control experiments were performed with KCl solutions, in this case the addition of 0.00001 M HCl to 0.1 M KCl had only a slight influence, a shift of 0.002 volt in one, or 0.006 volt in another case was observed, very much in contrast to the observations with  $\text{CaCl}_2$  solutions.

It appears, therefore, that the potential difference of a  $\text{CaCl}_2$  solution is dependent upon the diminutive traces of HCl formed by the interaction at the boundary and hence by the velocity with which these traces are washed away from the boundary, or by the purity of the water, or by neutralization by traces of basic salts, by the glass surface, etc. Hence it seems only natural that the effect of dilution should be very variable in the case of  $\text{CaCl}_2$  solutions on collodion, as described by Michaelis and Fujita (6), being even in the opposite direction in some cases according to their statements. For the same reason the average effect is sometimes smaller with  $\text{CaCl}_2$  even though an equally intensive chemical reaction occurs between dissolved  $\text{CaCl}_2$  and collodion as between KCl or NaCl and collodion.

In addition to the foregoing measurements, we performed experiments with KCl dissolved in a mixture of 50 per cent water and 50 per cent alcohol instead of pure water. With this solvent

mixture the potential difference varies likewise with the KCl concentration, as the following measurements show.

			volt	Difference volt
+ 0.1 M KCl	collodion	0.1 M KCl + alcohol*	- 0.002	
				0.028
- 0.1 M KCl	collodion	0.01 M KCl + alcohol*	+ 0.026	
				0.027
+ 0.1 M KCl	collodion	0.1 M KCl + alcohol*	- 0.001	

\* Equal volumes of alcohol and salt solution were mixed; hence the actual KCl concentration is 0.05 M or 0.005 M.

In this case also, we were able to demonstrate the formation of acid from collodion and KCl, by a comparative experiment, *viz.* by shaking celloidin cubes with either the solvent (water + alcohol) alone or with the solvent + KCl.

*Experiment 9 (by W. M. L.). 30 Cubes in Each 30 Cc. of Water and Alcohol (15 Cc. of Water + 15 Cc. of Ethyl Alcohol) or of Alcohol and Salt (15 Cc. of Ethyl Alcohol + 15 Cc. of 0.1 M KCl) — 10 cc. were titrated.*

0.40 cc. 0.01 N NaOH used for alcohol and 0.1 M KCl  
0.28 " 0.01 " " " " water and alcohol

Finally, analogous experiments were performed with water + alcohol + ether as solvent. The electromotive effect of concentration as well as acid formation was observed in this case, as in the previously mentioned examples.

Not only by titration, but also by pH measurements, is it possible to show that more acid is formed when a salt solution is shaken with collodion than after shaking pure water with collodion. This increased acidity can be demonstrated even by means of the rather crude colorimetric method, *viz.* by simply adding a suitable indicator to a KCl solution shaken with collodion and to water shaken in the same way. We selected as indicator brom-cresol green (La Motte) (pH 3.8 to 5.4), and proceeded as follows:

*Experiment 10 (by W. M. L.)*—60 cubes of celloidin (Schering) were shaken for 10 days with 20 cc. of a 0.1 M KCl solution; simultaneously 60 other celloidin cubes, as nearly identical with the former as possible, were shaken with 20 cc. of distilled water. The filtered aqueous solutions were used for pH determinations.

Considering that these solutions contain only KCl and traces of HCl, and hence are not all "buffered," it is advisable to use as little indicator as possible. Most indicators, including brom-cresol green, are acids which are used as sodium salts and hence neutralize some of the small amount of HCl present. Moreover, the solutions should be diluted as little as possible since this also lowers the acidity of an unbuffered solution. Nevertheless, it was necessary to equalize the KCl concentrations—which we did by adding 1 cc. of  $M$  KCl solution to 9 cc. of the water shaken with the collodion, and accordingly 1 cc. of  $0.1 M$  KCl was added to 9 cc. of the  $0.1 M$  KCl solution shaken with the collodion. 0.2 cc. of a 0.02 per cent brom-cresol green solution was added to the two samples of 10 cc. each thus obtained.

Even though this method is only semiquantitative a very marked distinction in color was visible, the solution made from the water shaken with the collodion appearing greenish, that from the KCl shaken with the collodion solution yellow. On comparing them with Walpole acetate buffer mixtures, the same amount of the same indicator being used, the former solution showed  $pH = 4.8$ , the latter  $pH = 3.8$ . These values do not constitute the accurate  $pH$  of the two solutions but are quoted merely in order to illustrate the magnitude of this differential effect.

In order to equalize these  $pH$  values 0.4 cc. of a  $0.01 N$  NaOH had to be added to the more acid solution. (If the precaution of equalizing the KCl concentrations after the shaking was omitted, the same difference in color was observed when the indicator was added, showing that the KCl influences the  $pH$  reading only imperceptibly, as one might expect.) We have repeated these experiments with essentially the same results.

#### SUMMARY

1. Collodion is not a chemically inert substance. It enters into chemical surface reactions with simple salt solutions in contact with it. HCl is thus formed while Na is taken up by the collodion.

2. These findings are difficult to reconcile with the theory of Michaelis according to which collodion is inert and potential differences following the variation of concentration are produced by dint of differential ionic mobility in the pores, the pores acting as ionic sieves.

On the basis of additional experiments, we expect to show that such chemical reactions at the phase boundary must necessarily be the cause of the observed electromotive forces (at collodion film) as well as of their variation with the salt concentration.

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# THE IONIZATION OF *dl*-ALANINE FROM TWENTY TO FORTY-FIVE DEGREES\*

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## INTRODUCTION

The need for accurate determinations of the ionization constants of the amino acids has been pointed out by Kirk and Schmidt (1). The common potentiometric method yields apparent or "hybrid" constants, which depend upon the ionic strength of the solution, and are subject to the inherent errors of cells with liquid junction. This may account for the discrepancies among the results reported by different investigators. Harned and coworkers (2) have developed a method of obtaining the true thermodynamic ionization constants of weak acids and bases from electromotive force measurements of suitable cells without liquid junction. A modification of this method has been applied in the present investigation to a representative amino acid, *dl*-alanine.

## EXPERIMENTAL

A synthetic preparation of *dl*-alanine (Pfanstiehl Chemical Company) was recrystallized from water and from 50 per cent alcohol. The product was dried in a vacuum desiccator. An exact determination of the moisture content was carried out in an air oven at 105° and checked in a modified Abderhalden dryer at 50°. 2.5 gm. of the material gave no weighable ash. The alanine was free from chloride and had a satisfactory nitrogen content as determined by the Kjeldahl method. Hydrochloric acid was carefully standardized by weighing as silver chloride. Sodium

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hydroxide solution, free from carbonate, was standardized against thoroughly dried potassium acid phthalate. Purified sodium chloride was dried by heating to 300°.

Stock solutions for determining the first acid constant were prepared by adding a weighed amount of alanine to a weighed amount of hydrochloric acid solution. Those for the second acid constant were made from weighed amounts of alanine, sodium hydroxide solution, and sodium chloride. These stock solutions were kept at 0° to prevent bacterial decomposition. Weighed amounts of the stock solutions were diluted with distilled water and freed from air by boiling *in vacuo* at 50°. Nitrogen was admitted to replace the air and the weight of the final solution determined. Cells of the simple H-type were filled by a vacuum technique to avoid contamination with air.

Tank hydrogen was purified at 500° by passing over copper reduced from the oxide. Well platinized platinum foil was used for the hydrogen electrodes. The silver-silver chloride electrodes were of the Type 2 described by Harned (3). Fresh electrodes were prepared for each solution measured. After careful washing with distilled water (the silver-silver chloride electrodes were soaked overnight), the electrodes were washed with three changes of the respective solutions over a period of 2 hours before being used in the cells.

The cells were placed in a well controlled constant temperature bath at 20° and kept at that temperature until equilibrium was attained. The temperature was then raised by successive 5° intervals, equilibrium of the electrodes being attained at each step. E.M.F. values of duplicate cells were thus obtained at six temperatures. After the highest temperature was reached, it was possible to check the electromotive force of the cells again at 20° within 0.1 millivolt or less, showing that during a period of several hours no progressive reaction at the electrodes had taken place.

A summary of the experimental data is given in Tables I and II. Three separate stock solutions were made up for each constant. These are represented by the three series of results. In Table I the first column represents the total molality of the hydrochloric acid, the second column the total molality of the alanine minus the molality of the hydrochloric acid, and the following columns the electromotive force measurements at the indicated tempera-

tures. In Table II the first column represents the total molality of the sodium hydroxide, the second column the total molality of the alanine minus the molality of the sodium hydroxide, the third column the molality of the sodium chloride, and the following

TABLE I

*Experimental Data Used in Determining First Acid Ionization Constant of dl-Alanine*

$T_{\text{HCl}}$	$T_{\text{R}} - T_{\text{HCl}}$	Observed $E$ , corrected to 1 atmosphere $\text{H}_2$ , at					
		20°	25°	30°	35°	40°	45°
0 007461	0 007645	0 50711	0 50846	0 50976	0 51096	0 51207	0 51309
0 01068	0 01094	0 49541	0 49652	0 49764	0 49863	0 49955	0 50036
0 01298	0 01330	0 48920	0 49017	0 49103	0 49193	0 49265	0 49358
0 02063	0 02114	0 47571	0 47639	0 47705	0 47773	0 47827	0 47883
0 02606	0 02670	0 46912	0 46973	0 47024	0 47075	0 47115	0 47151
0 04242	0 04347	0 45598	0 45633	0 45664	0 45691	0 45709	0 45718
0 06405	0 06563	0 44598	0 44616	0 44631	0 44646	0 44652	0 44648
0 08356	0 08562	0 43964	0 43969	0 43970	0 43975	0 43971	0 43966
0 01059	0 01059	0 49544	0 49661	0 49771	0 49872	0 49963	0 50046
0 01584	0 01585	0 48316	0 48408	0 48498	0 48576	0 48645	0 48710
0 03147	0 03148	0 46367	0 46420	0 46468	0 46512	0 46551	0 46585
0 04692	0 04694	0 45327	0 45360	0 45388	0 45412	0 45433	0 45450
0 06119	0 06122	0 44664	0 44687	0 44705	0 44718	0 44730	0 44738
0 07716	0 07720	0 44095	0 44108	0 44114	0 44117	0 44112	0 44101
0 02253	0 02256	0 47277	0 47343	0 47407	0 47464	0 47517	0 47567
0 04311	0 04317	0 45537	0 45572	0 45605	0 45637	0 45661	0 45682
0 07583	0 07594	0 44146	0 44156	0 44165	0 44173	0 44173	0 44172
0 08735	0 08748	0 43807	0 43814	0 43813	0 43805	0 43786	0 43766
0 11178	0 11194	0 43257	0 43254	0 43246	0 43237	0 43222	0 43204

$T$  = total molality.

columns the electromotive force measurements at the indicated temperatures.

### *First Acid Constant*

Buffer solutions of alanine and hydrochloric acid are measured in the cell





TABLE II  
Experimental Data Used in Determining Second Acid Ionization Constant of *dl*-Alanine

$T$ NaOH	$T_R - T$ NaOH	$m$ NaCl	Observed $E$ , corrected to 1 atmosphere $H_2$ , at					
			20°	25°	30°	35°	40°	45°
0 005696	0 005760	0 006093	0 93550	0 93643	0 93726	0 93798	0 93858	0 93898
0 007362	0 007445	0 007876	0 92971	0 93055	0 93129	0 93191	0 93237	0 93278
0 009474	0 009582	0 010136	0 92399	0 92469	0 92533	0 92582	0 92622	0 92653
0 01468	0 01484	0 01570	0 91359	0 91412	0 91457	0 91488	0 91510	0 91524
0 02205	0 02230	0 02358	0 90355	0 90393	0 90419	0 90437	0 90446	0 90445
0 02987	0 03021	0 03196	0 89636	0 89661	0 89675	0 89681	0 89677	0 89664
0 04561	0 04612	0 04879	0 88617	0 88624	0 88621	0 88606	0 88582	0 88548
0 06057	0 06125	0 06480	0 87957	0 87951	0 87936	0 87909	0 87874	0 87830
0 005169	0 004965	0 005168	0 93996	0 94097	0 94187	0 94269	0 94340	0 94402
0 006262	0 006015	0 006261	0 93672	0 93764	0 93850	0 93923	0 93981	0 94028
0 007676	0 007373	0 007675	0 93183	0 93268	0 93344	0 93410	0 93463	0 93507
0 01608	0 01544	0 01608	0 91417	0 91472	0 91514	0 91548	0 91573	0 91588
0 02353	0 02261	0 02353	0 90525	0 90564	0 90595	0 90612	0 90625	0 90621
0 03183	0 03058	0 03183	0 89787	0 89816	0 89826	0 89832	0 89832	0 89824
0 04767	0 04579	0 04766	0 88836	0 88844	0 88843	0 88832	0 88813	0 88781
0 01071	0 01067	0 01070	0 92283	0 92354	0 92413	0 92462	0 92499	0 92525
0 01507	0 01501	0 01505	0 91512	0 91567	0 91612	0 91648	0 91674	0 91689
0 02196	0 02188	0 02194	0 90595	0 90633	0 90664	0 90684	0 90692	0 90693
0 03080	0 03069	0 03078	0 89788	0 89815	0 89832	0 89838	0 89829	0 89811
0 04478	0 04462	0 04474	0 88914	0 88922	0 88921	0 88908	0 88890	0 88863
0 05742	0 05720	0 05736	0 88304	0 88305	0 88294	0 88272	0 88242	0 88206
0 07564	0 07526	0 07557	0 87680	0 87665	0 87642	0 87612	0 87570	0 87519

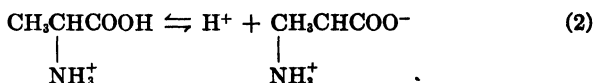
$T$  = total molality;  $m$ , molality.

which has an electromotive force defined by the equation

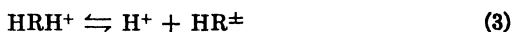
$$E = E_0 - 2.3026 \frac{RT}{nF} \log m_{H^+} m_{Cl^-} \gamma_{H^+} \gamma_{Cl^-} \quad (1)$$

where  $E_0$  is the potential of the cell when the hydrochloric acid is at unit activity.

In accordance with the views of Adams (4) and Bjerrum (5), and in line with the definition of an acid and base by Brönsted (6), alanine is considered as a zwitter ion, and its cation as a dibasic acid. Then the ionization of alanine in an acid solution is



This may be symbolized by



The true thermodynamic ionization constant is

$$K_{a_1} = \frac{a_{H^+} a_{HR^\pm}}{a_{HRH^+}} = \frac{m_{H^+} m_{HR^\pm} \gamma_{H^+} \gamma_{HR^\pm}}{m_{HRH^+} \gamma_{HRH^+}} \quad (4)$$

where  $a$  designates activity,  $m$  molality, and  $\gamma$  the activity coefficient. Since the solution is electrically neutral,

$$T_{Cl^-} = T_{HCl} = m_{HRH^+} + m_{H^+} \quad (5)$$

The total molality of alanine is equal to the sum of the molalities of the zwitter ion and the positive alanine ion.

$$T_R = m_{HR^\pm} + m_{HRH^+} \quad (6)$$

When Equations 5 and 6 are solved for the molalities of zwitter ion and positive alanine ion, the relations in Equations 7 and 8 are obtained.

$$m_{HRH^+} = T_{HCl} - m_{H^+} \quad (7)$$

$$m_{HR^\pm} = T_R - T_{HCl} + m_{H^+} \quad (8)$$

If Equations 7 and 8 are substituted in the logarithmic form of Equation 4, there is obtained

$$-\log K_{a_1} + \log \frac{\gamma_{H^+} \gamma_{HR^\pm}}{\gamma_{HRH^+}} = -\log m_{H^+} + \log \frac{T_{HCl} - m_{H^+}}{T_R - T_{HCl} + m_{H^+}} \quad (9)$$

The second term of Equation 9 is the logarithm of a ratio of the activity coefficients of 2 positive ions times the activity coefficient of a zwitter ion. According to the Debye-Hückel theory the activity coefficient of a single ion may be written

$$-\log \gamma = Az^2 \sqrt{\mu} / (1 + a \sqrt{\mu}) \quad (10)$$

where  $A$  is the theoretical Debye-Hückel constant,  $z$  the valence of the ion,  $\mu$  the ionic strength of the solution, and  $a$  an empirical constant proportional to the ionic diameter. If the indicated division in the right-hand member of the equation be carried out, the result is that

$$-\log \gamma = Az^2 \sqrt{\mu} + aAz^2 (-1 + a\mu^{\frac{1}{2}} - a^2\mu + \dots) \mu \quad (11)$$

$$-\log \gamma = Az^2 \sqrt{\mu} \pm \beta_0 \mu \quad (12)$$

The activity coefficient of the zwitter ion may be represented by

$$-\log \gamma_{\text{HR}\pm} = \pm \beta_1 \mu \quad (13)$$

The second term of Equation 9 when summed up for the activity coefficients of the 3 ions, becomes

$$\log (\gamma_{\text{H}^+} \gamma_{\text{HR}\pm} / \gamma_{\text{HRH}^+}) = (\mp \beta_2 \pm \beta_3 \mp \beta_1) \mu = \pm \beta \mu \quad (14)$$

where  $\beta$  is a complicated function of the ionic strength which, though essentially constant in the range of concentrations employed in the present investigation, need not be evaluated.

All the quantities on the right-hand side of Equation 9 are known except the molality of the hydrogen ion. An approximate value may be obtained in the following manner. Let

$$\log \gamma_{\text{H}^+} \gamma_{\text{Cl}^-} = -2A \sqrt{\mu} \quad (15)$$

Substitution of this relation in Equation 1 and rearrangement gives

$$m_{\text{H}^+} = 10^{-\frac{(E - E_0) nF}{2.3026 RT} - \log m_{\text{Cl}^-} + 2A \sqrt{\mu}} = 10^{\theta(E)} \quad (16)$$

By substitution of Equation 16 for  $m_{\text{H}^+}$ , Equation 9 may now be written

$$-\log K_{a_1} \pm \beta \mu = \frac{(E - E_0) nF}{2.3026 RT} + \log m_{\text{Cl}^-} - 2A \sqrt{\mu} + \log \frac{T_{\text{HCl}} - 10^{\theta(E)}}{T_{\text{R}} - 10^{\theta(E)}} \quad (17)$$

All of the terms in the right-hand side of Equation 17 are known and for convenience the right-hand side is represented by  $F_1$ , or

$$-\log K_{a_1} \pm \beta \mu = F_1 \quad (18)$$

An alternative procedure is to make the quite probable assumption that the activity of hydrochloric acid in the alanine buffer solutions is equal to that in pure hydrochloric acid at the same

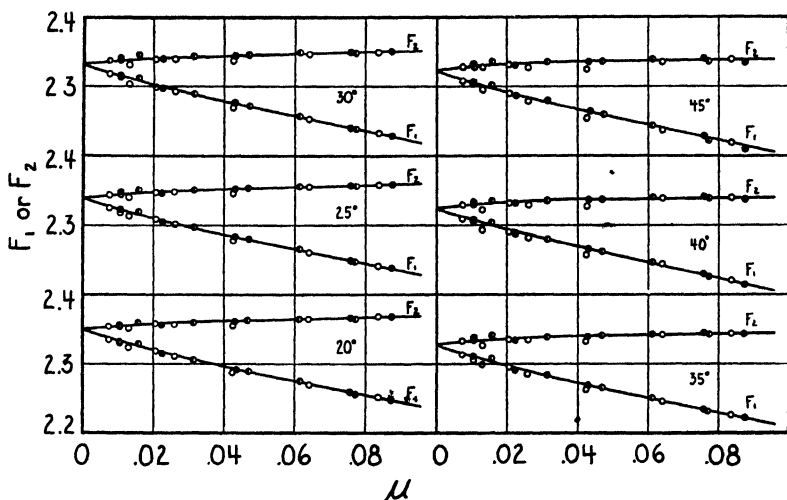


FIG. 1. The extrapolation which was used to obtain the first acid ionization constant of alanine. The values of  $F_1$  or  $F_2$  are plotted against the ionic strength and the intercept at zero ionic strength is the negative logarithm of  $K_{a_1}$ .  $\circ$  represents Series 1,  $\bullet$  Series 2, and  $\bullet$  Series 3.

ionic strength. Harned and Ehlers<sup>1</sup> have recently determined the activity coefficients of hydrochloric acid over a wide temperature range. Use may be made of these activity coefficients in Equation 1 to determine  $m_{H^+}$ . This leads to Equation 19

$$-\log K_{a_1} \pm \beta \mu = F_2 \quad (19)$$

where  $F_2$  again contains only terms that are known. To obtain  $-\log K_{a_1}$  a plot is made either of  $F_1$  or  $F_2$  against the ionic strength

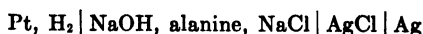
<sup>1</sup> The activity coefficients of hydrochloric acid used in the calculations were obtained privately from Professor H. S. Harned.

and extrapolated to zero ionic strength. The extrapolated value is the negative logarithm of the thermodynamic ionization constant.

Fig. 1 is the plot used to obtain the constants at the different temperatures. Either method of extrapolating the results gives the same value for the negative logarithm of the ionization constant.

### Second Acid Constant

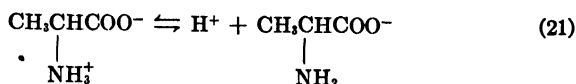
Buffer solutions of sodium hydroxide, sodium chloride, and alanine are measured in the cell



which has an electromotive force defined by the equation

$$E = E_0 - 2.3026 \frac{RT}{nF} \log a_{\text{H}^+} m_{\text{Cl}^-} \gamma_{\text{Cl}^-} \quad (20)$$

The second stage of the ionization of alanine (in an alkaline solution), and the corresponding ionization constant, may be written



$$K_{a_2} = \frac{a_{\text{H}^+} a_{\text{R}^-}}{a_{\text{HR}^\pm}} = \frac{a_{\text{H}^+} m_{\text{R}^-} \gamma_{\text{R}^-}}{m_{\text{HR}^\pm} \gamma_{\text{HR}^\pm}} \quad (23)$$

Analogous to Equations 5, 6, 7, and 8, the relations between the molal concentrations are

$$m_{\text{Na}^+} = T_{\text{NaOH}} = m_{\text{OH}^-} + m_{\text{R}^-} \quad (24)$$

$$T_{\text{R}} = m_{\text{HR}^\pm} + m_{\text{R}^-} \quad (25)$$

$$m_{\text{R}^-} = T_{\text{NaOH}} - m_{\text{OH}^-} \quad (26)$$

$$m_{\text{HR}^\pm} = T_{\text{R}} - T_{\text{NaOH}} + m_{\text{OH}^-} \quad (27)$$

If  $a_{\text{H}^+}$  is eliminated from Equations 20 and 23, and account is

taken of Equations 26 and 27, the resulting equation in logarithmic form is

$$-\log K_{a_1} + \log \frac{\gamma_R}{\gamma_{HR\pm} \gamma_{Cl^-}} = \frac{(E - E_0) nF}{2.3026 RT} + \log \frac{m_{Cl^-} (T_R - T_{NaOH} + m_{OH^-})}{T_{NaOH} - m_{OH^-}} \quad (28)$$

The second term on the left side of Equation 28 may be represented as

$$\log (\gamma_R / \gamma_{HR\pm} \gamma_{Cl^-}) = \pm \beta \mu \quad (29)$$

by proceeding as in Equations 9 to 14.  $m_{OH^-}$  in Equation 28 need only be estimated to an accuracy of 10 per cent in order to evaluate the right side with sufficient accuracy. The following method (suggested by Dr. D. I. Hitchcock) can be applied. The expression for the ionization constant of water is solved for  $m_{OH^-}$ . The numerator and the denominator of the resulting fraction are multiplied by the activity of the chloride ion. Since the solutions are quite dilute, the ratio of the activity coefficients of the chloride ion and the hydroxyl ion may be taken as equal to unity.

$$m_{OH^-} = \frac{K_w}{a_{H^+} \gamma_{OH^-}} = \frac{K_w m_{Cl^-} \gamma_{Cl^-}}{a_{H^+} a_{Cl^-} \gamma_{OH^-}} = \frac{K_w m_{Cl^-}}{a_{H^+} a_{Cl^-}} \quad (30)$$

The activity product of the hydrogen and the chloride ion is given explicitly by Equation 20.

The right-hand side of Equation 28 contains only known terms and for convenience is represented as  $F_3$ , resulting in the final equation

$$-\log K_{a_1} \pm \beta \mu = F_3 \quad (31)$$

If the values of  $F_3$  are plotted against the ionic strength, the intercept at zero ionic strength is the negative logarithm of the second ionization constant of alanine. Fig. 2 is the plot used to obtain the constants at the different temperatures. At low values of the ionic strength the curve begins to fall off quite sharply. This is

possibly due to the dissolving of silver chloride to form complex salts of alanine in these alkaline solutions. In the very dilute solutions this would increase the effective chloride ion concentrations and make the observed electromotive forces too low, thus giving too low a value for  $-\log K_a$  if the curve were bent down to follow these low points. To avoid this possible error, the points below  $0.015\mu$  have been neglected in performing the extrapolation.

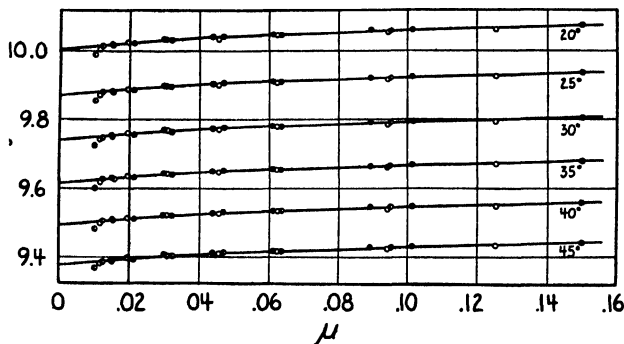


FIG. 2. The extrapolation which was used to obtain the second acid ionization constant of alanine. The value of  $F_2$  is plotted against the ionic strength and the intercept at zero ionic strength is the negative logarithm of  $K_{a2}$ .  $\circ$  represents Series 1,  $\bullet$  Series 2, and  $\bullet$  Series 3.

#### DISCUSSION

Table III gives the final results for the negative logarithms of the constants, the  $E_0$  values (7) for the type of cell used, the theoretical Debye-Hückel constant  $A$ , and the heats of ionization calculated by the formula

$$\Delta H = 2.30RT^2 d \log K/dT \quad (32)$$

where  $d \log K/dT$  was obtained by a graphical analytical method.

The previously reported values for the first acid ionization constant are in quite good agreement with the values reported here. The older values for the second acid constant, expressed as  $pK$ , are about 0.1 unit lower than those reported here (1, 8). Bjerrum and Unmack (9), using cells with a liquid junction and a similar extrapolation method, have determined the ionization

constants of glycine. Their value for the second ionization constant of glycine also differs in the same direction from the previously reported values. The accuracy attained in determining the constants in this investigation is believed to be  $\pm 0.003$  pK, and the constants may be translated into the classical constants by the proper use of the ionization constant of water. The first acid constant changes but little with temperature and seems to be approaching a maximum (minimum value in pK units) in the neighborhood of  $50^\circ$ , while the second shows quite a marked change with temperature.

TABLE III  
*Values of  $\Delta H$ ,  $-\log K_{a_1}$ , and  $-\log K_{a_2}$  at Various Temperatures*

Temperature °C	$E_0$	$A$	$-\log K_{a_1}$	$\Delta H_1$	$-\log K_{a_2}$	$\Delta H_2$
20	0 22554	0 502	2 350	913	10 006	10900
25	0 22239	0 506	2 340	720	9 870	10810
30	0 21908	0 511	2 332	538	9 740	10740
35	0 21561	0 516	2 327	369	9 615	10680
40	0 21200	0 522	2 324	215	9 494	10630
45	0 20825	0 528	2 322	79	9 378	10580

#### SUMMARY

1. The method of determining the ionization constants of weak acids by means of cells without liquid junction has been shown to be applicable to a representative amino acid, *dl*-alanine.

2. The values of the negative logarithms of the first and second ionization constants of *dl*-alanine have been determined from  $20$ – $45^\circ$  with an accuracy greater than heretofore possible.

3. From the variation of the logarithms of the constants with the temperature, the heats of ionization of *dl*-alanine have been calculated.

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## CONFIGURATIONAL RELATIONSHIP OF ISOPROPYL-CARBINOLS

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(Received for publication, April 28, 1933)

On the basis of indirect evidence the relationship between the two homologous series of the *n*-propyl- and of the isopropyl secondary carbinols given in Table I was suggested.<sup>1</sup> By direct chemical methods, one of the series, namely the methylisopropylcarbinol, has been correlated by Stevens<sup>2</sup> to the corresponding methyl-*n*-propylcarbinol. Naturally, it was desirable to correlate by direct chemical methods the higher members of the isopropyl series to the corresponding members of the propyl series. In the present communication are presented the results of the correlation of ethylisopropylcarbinol to ethylpropylcarbinol. The procedure is presented in Table II, in which the present results are compared with those of Stevens. From Table II it may be seen that also in the case of ethylisopropylcarbinol the direct chemical method leads to the same conclusion as the indirect method. This is important not only in its application to this special case but for the reason that it furnishes evidence for the reliability of the indirect method employed by Levene and Marker,<sup>1</sup> a method which has been applied also in the case of the series of cyclohexyl secondary carbinols.

### EXPERIMENTAL

*Dextro-Hydroxybutyric Acid and Its Ester*—The dextro- $\alpha$ -hydroxybutyric acid and its corresponding ester were prepared by

<sup>1</sup> Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **90**, 669 (1931); **97**, 379 (1932). Freudenber, K., *Sitzungsber. Heidelberg. Akad. Wissensch., Math.-Naturwissensch. Kl.*, 9 (1931).

<sup>2</sup> Stevens, P. G., *J. Am. Chem. Soc.*, **54**, 3732 (1932).

way of resolution of the 2-bromobutyric acid, which was converted into the amino acid and this deaminized into the hydroxybutyric acid. It was found less time-consuming to prepare a sufficient

TABLE I  
Maximum Molecular Rotations of Carbinols and Phthalates

n-Propyl series		Isopropyl series	
Carbinol	Phthalate (in alcohol)	Carbinol	Phthalate (in alcohol)*
$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{OH} \\   \\ \text{C}_2\text{H}_7 \text{ (n)} \\ +12.1 \end{array}$	+90.0	$\begin{array}{c} \text{CH}_3 \\   \\ \text{HC}-\text{OH} \\   \\ \text{C}_2\text{H}_7 \text{ (iso)} \\ +4.7 \end{array}$	+98
$\begin{array}{c} \text{C}_2\text{H}_5 \\   \\ \text{HC}-\text{OH} \\   \\ \text{C}_2\text{H}_7 \text{ (n)} \\ +5.1 \end{array}$	+18.7	$\begin{array}{c} \text{C}_2\text{H}_5 \\   \\ \text{HC}-\text{OH} \\   \\ \text{C}_2\text{H}_7 \text{ (iso)} \\ -16.7 \end{array}$	+10
$\begin{array}{c} \text{C}_2\text{H}_7 \text{ (n)} \\   \\ \text{HC}-\text{OH} \\   \\ \text{C}_2\text{H}_7 \text{ (n)} \\ 0 \end{array}$	0	$\begin{array}{c} \text{C}_2\text{H}_7 \text{ (n)} \\   \\ \text{HC}-\text{OH} \\   \\ \text{C}_2\text{H}_7 \text{ (iso)} \\ -27.1 \end{array}$	-22
$\begin{array}{c} \text{C}_4\text{H}_9 \text{ (n)}^\dagger \\   \\ \text{HC}-\text{OH} \\   \\ \text{C}_2\text{H}_7 \text{ (n)} \\ -0.95 \end{array}$	-6.7	$\begin{array}{c} \text{C}_4\text{H}_9 \text{ (n)} \\   \\ \text{HC}-\text{OH} \\   \\ \text{C}_2\text{H}_7 \text{ (iso)} \\ -35.9 \end{array}$	-36
$\begin{array}{c} \text{C}_6\text{H}_{11} \text{ (n)} \\   \\ \text{HC}-\text{OH} \\   \\ \text{C}_2\text{H}_7 \text{ (n)} \\ \text{Levo} \end{array}$	Levo	$\begin{array}{c} \text{C}_6\text{H}_{11} \text{ (n)} \\   \\ \text{HC}-\text{OH} \\   \\ \text{C}_2\text{H}_7 \text{ (iso)} \\ -38.2 \end{array}$	-44

\* Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **103**, 1957 (1913).

† Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 418 (1931).

quantity of the starting material in this manner rather than by the resolution of the hydroxy acid. The details of the preparation will be presented in another article.

*Levo-Ethyl Ester of  $\alpha$ -Ethoxybutyric Acid*—40 gm. of levo-ethyl

TABLE II  
Establishment of Configurational Relationship of Methylisopropylcarbinol to Ethylisopropylcarbinol.  $[M]_D^{25}$  (Not Maximum Rotation)

$\text{CH}_3$ $\text{HC}-\text{OH}$ $\text{C}_2\text{H}_7$ (n)	$\text{CH}_3$ $\text{HC}-\text{OH}$ $\text{COOH}$	$\text{CH}_3$ $\text{HC}-\text{OH}$ $\text{COOC}_2\text{H}_5$	$\text{CH}_3$ $\text{HC}-\text{OC}_2\text{H}_5 \rightarrow$ $\text{COOC}_2\text{H}_5$	$\text{CH}_3$ $\text{HC}-\text{OC}_2\text{H}_5 \rightarrow$ $\text{COH}$	$\text{CH}_3$ $\text{HC}-\text{OC}_2\text{H}_5 \rightarrow$ $\text{C}$ $\text{CH}_3, \text{CH}_2$ -2.1§	$\text{CH}_3$ $\text{HC}-\text{OC}_2\text{H}_5 \rightarrow$ $\text{CH}$ $\text{CH}_3, \text{CH}_2$ +1.4§	$\text{CH}_3$ $\text{HC}-\text{OH}$ $\text{CH}$ $\text{CH}_3, \text{CH}_2$ Dextro§
Dextro*	Dextro* †	-0.6§	-5.1§	+2.4§	-2.1§	+1.4§	
$\text{C}_2\text{H}_5$ $\text{HC}-\text{OH}$ $\text{C}_2\text{H}_7$ (n)	$\text{C}_2\text{H}_5$ $\text{HC}-\text{OH}$ $\text{COOH}$	$\text{C}_2\text{H}_5$ $\text{HC}-\text{OH}$ $\text{COOC}_2\text{H}_5$	$\text{C}_2\text{H}_5$ $\text{HC}-\text{OC}_2\text{H}_5 \rightarrow$ $\text{COOC}_2\text{H}_5$	$\text{C}_2\text{H}_5$ $\text{HC}-\text{OC}_2\text{H}_5 \rightarrow$ $\text{C}-\text{OH}$ $\text{CH}_3, \text{CH}_2$ -12.1	$\text{C}_2\text{H}_5$ $\text{HC}-\text{OC}_2\text{H}_5 \rightarrow$ $\text{C}$ $\text{CH}_3, \text{CH}_2$ -22.8	$\text{C}_2\text{H}_5$ $\text{HC}-\text{OC}_2\text{H}_5 \rightarrow$ $\text{CH}$ $\text{CH}_3, \text{CH}_2$ -3.6 -6.5	$\text{C}_2\text{H}_5$ $\text{HC}-\text{OH}$ $\text{CH}$ $\text{CH}_3, \text{CH}_2$ -9.8
Dextro	Dextro	Levo	-58.3	-12.1	-22.8	-3.6 -6.5	-9.8

\* Levene, P. A., Haller, H. L., and Walti, A., *J. Biol. Chem.*, **72**, 591 (1927).

† Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **74**, 343 (1927).

§ Stevens, P. G., *J. Am. Chem. Soc.*, **54**, 3732 (1932).

ester of  $\alpha$ -hydroxybutyric acid (from dextro acid) were dissolved in 168 gm. of ethyl iodide and 125 gm. of dry silver oxide added in 10 gm. quantities at intervals of 15 minutes with stirring. After this had been added, 81 gm. of ethyl iodide were added and the product refluxed with stirring for several hours. The product was extracted with ether, dried, and distilled. B p  $67^\circ$  at 12 mm.

$$[\alpha]_D^{25} = \frac{-34.3^\circ}{1 \times 0.941} = -36.45^\circ, \quad [M]_D^{25} = -58.35^\circ \text{ (homogeneous)}$$

4.551 mg. substance    9.800 mg.  $\text{CO}_2$  and 3.905 mg.  $\text{H}_2\text{O}$   
 $\text{C}_8\text{H}_{18}\text{O}_3$     Calculated    C 60.0, H 10.1  
                  Found            " 58.7, " 9.6

*Levo-3-Ethoxy-4-Methylpentanol-4*—A Grignard reagent was prepared from 200 gm. of methyl iodide and 30 gm. of magnesium in dry ether. The reagent was cooled and 80 gm. of ethyl ester of  $\alpha$ -ethoxybutyric acid,  $[M]_D^{25} = -58.35^\circ$  (homogeneous), were slowly dropped in. The product was decomposed by pouring onto ice and ammonium chloride solution. The carbinol was extracted with ether and fractionated. B p  $162\text{--}163^\circ$  at 760 mm. Yield 45 gm.

$$[\alpha]_D^{25} = \frac{-7.37^\circ}{1 \times 0.886} = -8.3^\circ, \quad [M]_D^{25} = -12.2^\circ \text{ (homogeneous)}$$

3.605 mg. substance    8.545 mg.  $\text{CO}_2$  and 3.885 mg.  $\text{H}_2\text{O}$   
 $\text{C}_8\text{H}_{18}\text{O}_2$     Calculated    C 65.7, H 12.4  
                  Found            " 64.6, " 12.1

*Levo-3-Ethoxy-4-Methylpentene-5*—This carbinol was dehydrated by the Tschugaeff<sup>3</sup> method according to the technique used by Stevens<sup>2</sup> for the dehydration of 2-ethoxy-3-methylbutanol-3.

45 gm. of 3-ethoxy-4-methylpentanol-4,  $[M]_D^{25} = -12.2^\circ$  (homogeneous), were dissolved in 500 cc. of purified cymene. This was boiled for 3 hours with 20 gm. of clean potassium. 50 cc. of solvent were distilled off to remove unchanged carbinol and the excess potassium was then removed mechanically. The product was cooled, diluted with 500 cc. of dry ether, and 47 gm. of carbon disulfide were added. The solution was allowed to stand at room

<sup>3</sup> Tschugaeff, L., *Chem. Zentr.*, **1**, 94 (1905)

temperature for 1 hour and then 110 gm. of methyl iodide were added and the mixture placed in the hood overnight. The next day it was refluxed for 4 hours after which as much ether as possible was distilled off on a steam bath. The distillation was then continued in a metal bath until the temperature reached 170°. The distillate was washed successively with 5 per cent potassium hydroxide solution, mercuric chloride, water, and finally with potassium carbonate solution. It was dried and then fractionated.

The product had a boiling point of 120–130°.

$$[\alpha]_D^{25} = \frac{-14.5^\circ}{1 \times 0.815} = -17.8^\circ; [M]_D^{25} = -22.8^\circ \text{ (homogeneous)}$$

4.610 mg. substance: 12.675 mg. CO<sub>2</sub> and 5.270 mg. H<sub>2</sub>O

C<sub>8</sub>H<sub>16</sub>O. Calculated. C 74.9, H 12.6

Found. " 75.0, " 12.8

*Levo-2-Methyl-3-Ethoxypentane*—12 gm. of 3-ethoxy-4-methylpentene-5,  $[M]_D^{25} = -22.8^\circ$  (homogeneous), were dissolved in 25 cc. of glacial acetic acid. 1 gm. of platonic oxide was added and the mixture was shaken with hydrogen under a pressure of 45 pounds per sq. inch. Reduction reached completion in 1 hour. The mixture was allowed to shake overnight and the product was isolated as usual. B. p. 126°.

$$[\alpha]_D^{25} = \frac{-2.25^\circ}{1 \times 0.810} = -2.78^\circ; [M]_D^{25} = -3.61^\circ \text{ (homogeneous)}$$

4.770 mg. substance: 12.875 mg. CO<sub>2</sub> and 5.895 mg. H<sub>2</sub>O

C<sub>8</sub>H<sub>18</sub>O. Calculated. C 73.8, H 13.9

Found. " 73.6, " 13.8

*Dextro-2-Methyl-3-Ethoxypentane*—Inactive ethylisopropylcarbinol was converted into the half phthalate ester and resolved by crystallization of the strychnine salt from alcohol.

20 gm. of ethylisopropylcarbinol,  $[M]_D^{18} = +9.8^\circ$  (homogeneous), were dissolved in 300 cc. of cymene and 8 gm. of clean potassium were added. The product was refluxed until practically all the potassium had disappeared. Then 50 cc. of cymene were distilled off to remove unchanged carbinol. The solution was cooled, 40 gm. of ethyl iodide were added, and the mixture was refluxed for 1 hour. The product was now distilled until the temperature

reached 173°, the distillate shaken with a small amount of phosphorus anhydride and fractionated. The portion boiling at 120–130° was refluxed for 2 hours with metallic potassium, then fractionated. B. p. 124–126° at 760 mm. Yield 10 gm.

$$[\alpha]_D^{25} = \frac{+4.05^\circ}{1 \times 0.810} = +5.00^\circ; \quad [M]_D^{25} = +6.51^\circ \text{ (homogeneous)}$$

3.925 mg. substance: 10.625 mg. CO<sub>2</sub> and 4.970 mg. H<sub>2</sub>O

C<sub>8</sub>H<sub>18</sub>O. Calculated. C 73.8, H 13.9

Found. " 73.8, " 14.2

# THE RIBOSEPHOSPHORIC ACID FROM YEAST ADENYLIC ACID

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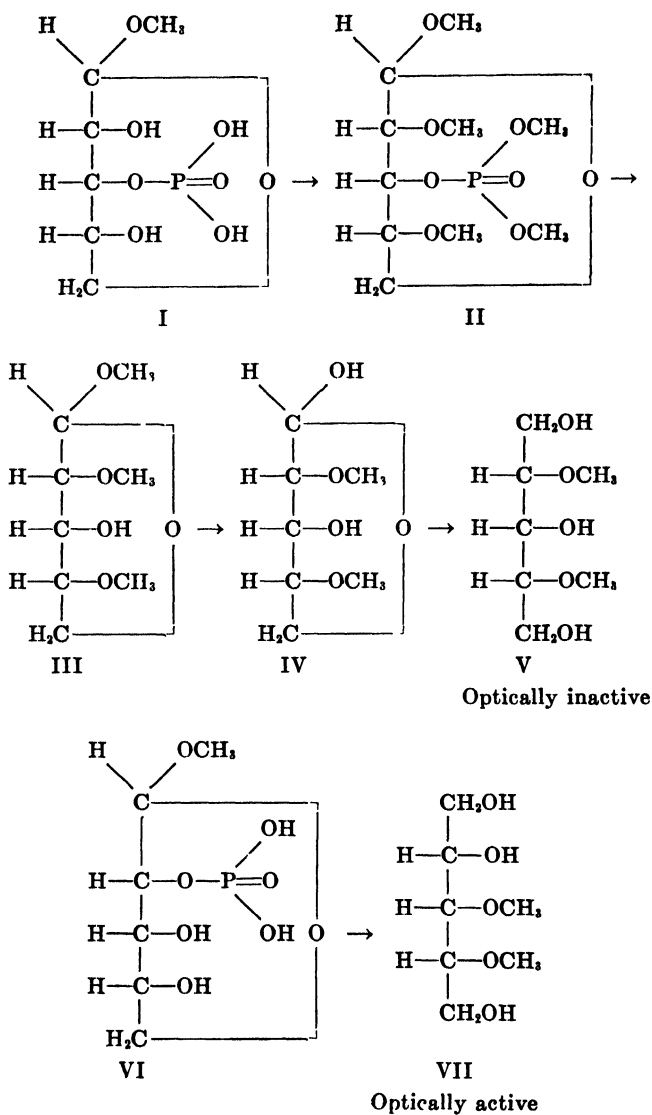
(Received for publication, May 1, 1933)

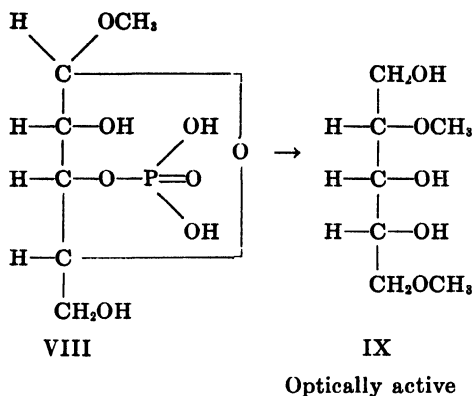
The problem of the position of the phosphoric acid in the molecule of guanylic acid required for its solution three steps: first, deamination of guanylic acid to xanthylic acid; secondly, hydrolysis of xanthylic acid to ribosephosphoric acid;<sup>1</sup> and thirdly, the reduction of the latter to *d*-ribitol-3-phosphoric acid.<sup>2</sup> The difficulties encountered by previous workers in converting yeast adenylic acid to the corresponding inosinephosphoric acid stimulated us to attempt to solve the problem of the position of the phosphoric acid residue in yeast adenylic acid without resorting to the process of deamination. In fact, it was possible, by passing dry hydrogen chloride through a suspension of yeast adenylic acid in dry methyl alcohol, to obtain the phosphoric ester of methylriboside. We were thus encouraged to continue the effort to locate the position of the phosphoric acid in the molecule of this substance. The problem seemed accessible of solution inasmuch as on the basis of the conditions during the formation of glycoside it was expected to obtain the pyranose glycoside. The following steps were envisaged for the solution of the problem. Firstly, exhaustive methylation of the ribosidephosphoric acid; secondly, hydrolysis of the phosphoric acid residue; thirdly, hydrolysis of dimethyl methylriboside to dimethyl ribose; and fourthly, reduction of the latter to dimethyl ribitol. The structure of the latter should have revealed the position of the phosphoric acid residue in the parent substance as can be seen from the following formulæ.

<sup>1</sup> Levene, P. A., and Harris, S. A., *J. Biol. Chem.*, **95**, 755 (1932).

<sup>2</sup> Levene, P. A., and Harris, S. A., *J. Biol. Chem.*, **98**, 9 (1932).





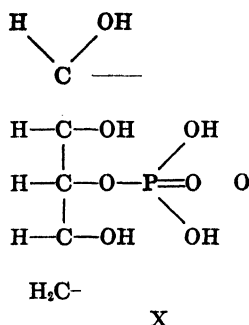


From these formulæ it can be seen that only substance (I) leads to an inactive dimethyl ribitol (V).

All the steps outlined in the transformation of (I) to (V) were successfully carried out. Unfortunately, at the end it was discovered that the original material contained a small proportion of substance (VIII). True, a method of removing the dimethyl methylribofuranoside from the dimethyl methylribofuranoside could be devised and in fact was devised, but this task required the preparation of a considerable quantity of the mixed glycosides, an undertaking both time-consuming and expensive. It was therefore decided to leave this task in abeyance for a time, and to attempt to solve the problem of the structure of yeast adenylic acid by the procedure which has been successfully employed in the case of guanylic acid.<sup>1,2</sup>

It has been found that yeast adenylic acid is readily deaminized into inosinephosphoric acid and this in turn is readily hydrolyzed in aqueous solution at its own pH to a ribosephosphoric acid. This acid was then compared with that obtained from xanthylic acid and the two were found identical with respect to their rotation in aqueous solution, with respect to the effect of the presence of borax on their optical activity, and finally, with respect to their rates of hydrolysis by mineral acids. Thus, the ribosephosphoric acid obtained from yeast adenylic acid is *d*-ribose-3-phosphoric acid (X).

## Ribosephosphoric Acid



Yeast adenylic acid then should be designated 1-adenine-*d*-ribofuranoside-3-phosphoric acid.<sup>3</sup> For convenience it may be referred to as adenosine-3-phosphoric acid in distinction to muscle adenylic acid which may be designated as adenosine-5-phosphoric acid. The analogous designations may be employed in the case of the two corresponding inosinic acids.

## EXPERIMENTAL

*Preparation of Yeast Adenylic Acid*—Adenylic acid was prepared according to the directions of Jones and Perkins<sup>4</sup> with the exception that the solution of the combined nucleotides was neutralized with ammonium hydroxide before evaporation. After removal of the guanylic acid, the adenylic acid was obtained by direct crystallization from the mixed nucleotides. One recrystallization from water yielded a very pure product, as shown by rotation and analysis.

*Hydrolysis of Adenylic Acid with Methyl Alcoholic Hydrogen Chloride*—A suspension of 2 gm. of adenylic acid in 25 cc. of methyl alcohol was treated with dry hydrogen chloride. In a moment the adenylic acid was completely dissolved and after 15 minutes adenine hydrochloride crystallized out. At the end of 30 minutes the bubbling of the gas was stopped and the reaction mixture was allowed to stand overnight in the refrigerator. The precipitate was removed by filtration and the filtrate poured into 5 times its volume of ice water. The hydrochloric acid was removed by shaking with moist silver carbonate. After precipitation of the silver with hydrogen sulfide the aerated filtrate was

<sup>3</sup> Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **94**, 809 (1931-32).

<sup>4</sup> Jones, W., and Perkins, M. E., *J. Biol. Chem.*, **62**, 557 (1924-25).

neutralized with barium hydroxide. The solution was evaporated to a dry mass which was washed with acetone and ether. This product was free from nitrogen, contained organic phosphorus, and was non-reducing. Analysis showed that there was approximately one methoxyl group present. A similar product was prepared from ammonium guanylate. However, a direct comparison of the two glycosides on the basis of their rotations is not permissible because of the possibility of different equilibria between  $\alpha$  and  $\beta$  forms. Also, the hydrolysis of the glycosidic group was accompanied by the hydrolysis of phosphorus, so this procedure was abandoned too.

*Methylation of Phosphomethylriboside from Adenylic Acid*—A suspension of 30 gm. of adenylic acid in 300 cc. of methyl alcohol was treated with dry hydrogen chloride for 30 minutes. After standing overnight in the refrigerator, the precipitate was filtered off and washed thoroughly with methyl alcohol. The filtrate was evaporated to a syrup, taken up in methyl alcohol, and the distillation repeated twice. The remainder of the hydrogen chloride was removed with a slight excess of dry silver oxide. This mixture was then treated with 50 cc. of methyl iodide, whereupon a spontaneous refluxing took place and continued until all of the silver was converted to silver iodide. The mixture was then filtered, and the silver residue thoroughly extracted with methyl alcohol. The syrup obtained by evaporation of the combined filtrates was methylated three times by the Purdie method. The methylated syrup was dissolved in ether and filtered to remove the basic material that had separated. After removal of the ether the syrup was distilled under reduced pressure. The yield was 10.5 gm. (40.5 per cent of the theoretical).  $n_D^{25} = 1.4500$ . The specific rotation was as follows:

$$[\alpha]_D^{25} = \frac{-5.75^\circ \times 100}{10.87 \times 2} = -26.4^\circ \text{ (in absolute alcohol)}$$

The analysis indicated a composition agreeing with that of a tetramethylphosphomethylriboside.

5 030 mg. substance:	7 325 mg. CO <sub>2</sub> and 3 300 mg H <sub>2</sub> O
3 750 " " "	: 14 015 " AgI
3 500 " " "	: 24 420 " ammonium phosphomolybdate
C <sub>10</sub> H <sub>21</sub> O <sub>6</sub> P	Calculated C 40 00, H 7 00, OCH <sub>3</sub> 51 6, P 10 32
	Found. " 39 71, " 7 34, " 49 3, " 10 13

A similar product was obtained (in a yield of 42.4 per cent) from 34 gm. of ammonium guanylate. It had also been obtained previously by the methylation of the ribosephosphoric acid from xanthylic acid.<sup>1</sup>

*Dephosphorylation of Methylated Phosphoriboside*—The methylated phosphoriboside from guanylic acid was dephosphorylated by heating 4 gm. in 100 cc. of methyl alcohol solution containing 3 equivalents of barium methyrate for 2 hours at 100°. The white precipitate formed during the reaction was nearly free of sugar and it gave no test for free phosphoric acid. The alcohol was removed by distillation and then the methylated sugar was extracted from the residue with ether. The precipitate was removed by centrifuging. The ether solution was free from phosphorus compounds but still contained barium methyrate. It was therefore evaporated to a syrup, dissolved in water, and the barium exactly removed with sulfuric acid. After filtration it was evaporated to dryness and distilled under reduced pressure. From 25.7 gm. of starting material 12 gm. (72.7 per cent yield) of distilled dimethyl methylriboside were obtained (b.p. 90–93° at less than 1 mm.). The analysis was as follows:

6 155 mg. substance:	11 240 mg. CO <sub>2</sub> and	4 480 mg. H <sub>2</sub> O
3 725 " " "	13 583 " AgI	
C <sub>8</sub> H <sub>18</sub> O <sub>8</sub> .	Calculated.	C 49 96, H 8 39, OCH <sub>3</sub> 48 45
	Found.	" 49 79, " 8 13, " 48 13

The specific rotation was as follows:

$$[\alpha]_D^{25} = \frac{-19.63^\circ \times 100}{20.17 \times 2} = -48.7^\circ \text{ (in methyl alcohol)}$$

$$[\alpha]_D^{25} = \frac{-19.60^\circ \times 100}{20.66 \times 2} = -47.4^\circ \text{ (in water)}$$

A sample prepared from adenylic acid had the following rotation.

$$[\alpha]_D^{25} = \frac{-14.34^\circ \times 100}{12.84 \times 2} = -55.8^\circ \text{ (in water)}$$

*Hydrolysis of Dimethyl Methylriboside*—The rate of hydrolysis was followed by observing the change in rotation in a test sample. The specific rotation (initially  $-41.3^\circ$ ) was practically constant

( $-13.75^{\circ}$ ) after heating for 90 minutes on the steam bath in 6 per cent aqueous hydrochloric acid. For preparation in larger quantities, 5 gm. of the glycoside in 100 cc. of the 6 per cent acid were heated for 2 hours at  $89^{\circ}$ . The solution was cooled in ice water and the acid removed with wet silver carbonate. The solution was evaporated to a thin syrup under reduced pressure. This was dissolved in chloroform and the solution dried with anhydrous sodium sulfate, filtered, evaporated, and the residue distilled (b.p.  $132-136^{\circ}$  at less than 1 mm. pressure). The yield from 12 gm. of starting material was 8 gm. (72 per cent of the theoretical). The refractive index was  $n_D^{25} = 1.4750$ . The specific rotation in water containing a drop of ammonia was as follows:

$$[\alpha]_D^{25} = \frac{-3.66^{\circ} \times 100}{10.98 \times 2} = -16.7^{\circ}$$

*Catalytic Reduction of Dimethyl Ribose*—The dimethyl ribose was reduced with Adams' catalyst<sup>5</sup> in solution under a pressure of 3 atmospheres of hydrogen. 6 gm. of sugar in 50 cc. of water were reduced in  $2\frac{1}{2}$  days with a total of 2 gm. of catalyst added in portions of 0.5 gm., the reduction being followed by the Willstätter titration as modified by Levene, Raymond, and Dillon.<sup>6</sup> A small amount of non-reduced sugar was now removed by oxidation and the main product extracted with acetone from the barium salt which was formed. The acetone solution was evaporated to dryness and the product acetylated. This material distilled at  $136-137^{\circ}$  at less than 1 mm. The specific rotation of four successive fractions changed from  $-1.55^{\circ}$  to  $+1.24^{\circ}$ . The methoxyl value was too high, showing that all of the glycoside had not been hydrolyzed.

On searching for other sources of impurity it was found that the original dimethyl methylriboside was not a pure glycoside but contained up to 20 per cent of the  $\gamma$  form. At this point the further investigation of this problem was postponed while the hydrolysis of adenylic acid was attacked from a different angle.

*Deaminization of Adenylic Acid*—10 gm. of adenylic acid were

<sup>5</sup> Adams, R., Voorhees, B., and Shriner, R. L., in Gilman, H., *Organic syntheses*, New York, coll. 1, 452 (1932).

<sup>6</sup> Levene, P. A., Raymond, A. L., and Dillon, R. T., *J. Biol. Chem.*, **95**, 699 (1932).

suspended in water and sufficient alkali was added to effect solution. To this were added 60 cc. of glacial acetic acid and to this solution 50 cc. of a 30 per cent potassium nitrite solution were added in a slow stream with constant mechanical stirring. The stirring was continued until gas evolution ceased (about 2 hours). The solution was allowed to stand another hour and then a 25 per cent lead acetate solution was added to it until precipitation was completed. The lead salt was filtered off and washed four times by stirring with warm water. The lead was removed by passing hydrogen sulfide through the suspension. Purification by this method was repeated. The solution containing only the free acid was evaporated to 30 cc. On addition of an equal volume of absolute alcohol the product settled out. This precipitate was washed with alcohol and ether, and dried in a vacuum desiccator. The mother liquor, on evaporation, yielded a further crop of semicrystalline product. The total yield was 5.6 gm. (56 per cent of the theoretical). This acid is much more soluble in water than the parent adenylic acid. With thymol blue indicator, the pH of its aqueous solution was shown to be about 1.6 to 1.7 while the pH of a solution of adenylic acid is about 3.5. The specific rotation was as follows:

$$[\alpha]_D^{25} = \frac{-2.14^\circ \times 100}{2.614 \times 2} = -41.0^\circ \text{ (in water)}$$

The composition agreed with the theoretical for inosine-3-phosphoric acid.

5 500 mg. substance:	7 100 mg	CO <sub>2</sub> and 1 950 mg. H <sub>2</sub> O
3 825 "	23 090 "	ammonium phosphomolybdate
0 1000 gm. "	11 24 cc	0 1000 N HCl (Kjeldahl)
C <sub>10</sub> H <sub>13</sub> N <sub>4</sub> O <sub>8</sub> P.	Calculated	C 34.4, H 3.74, P 8.91, N 16.10
	Found.	" 35.20, " 3.91, " 8.76, " 15.74

*Hydrolysis of Inosinephosphoric Acid from Adenylic Acid*—In previous papers,<sup>1,7</sup> the rate of hydrolysis of the different nucleotides to sugars was determined by the aid of the Hagedorn and Jensen titration. The estimation of the amount of sugar formed

<sup>7</sup> Levene, P. A., and Dmochowski, A., *J. Biol. Chem.*, **93**, 563 (1931).

was based on the previously determined value for free ribose. It was found that the titration factor for ribosephosphoric acid is 2.5 times lower than it is for free ribose.

Preliminary experiments were made to determine the approximate conditions for the formation of a ribosephosphoric acid from the new phosphinosine. Unlike xanthylic acid, this new acid in aqueous solution showed very little hydrolysis when heated at 50° for 2 days. However, it was about 80 per cent hydrolyzed at 95–100° in an hour, although there was a simultaneous hydrolysis of phosphorus to the extent of 20 per cent.

On the basis of this information hydrolyses were carried out for the preparation of the ribosephosphoric acid. 4.7 gm. of inosine phosphoric acid were dissolved in 200 cc. of water and the solution heated at 95–97° for 1 hour. It was then cooled in ice water and treated with acid mercuric sulfate solution (10 per cent) until no more precipitation took place. The mercury precipitate was filtered off and the filtrate was neutralized with barium carbonate. The mercury was removed with hydrogen sulfide. After aeration the solution was made faintly alkaline to phenolphthalein and filtered with charcoal. The volume was reduced by evaporation under diminished pressure to 40 cc. and then an equal volume of absolute alcohol was added to the solution. The yield of the dry crude salt was 1.1 gm. (22.4 per cent of the theoretical).

Purification of this product was accomplished in the following manner. 3 gm. of the crude barium ribosephosphate were dissolved in 100 cc. of water and centrifuged to remove the insoluble portion. Barium hydroxide was added until there was no more precipitation of barium phosphate. The precipitate was removed by filtration, the solution exactly neutralized with sulfuric acid, and then made faintly alkaline to phenolphthalein with barium hydroxide. After removal of the barium sulfate the solution was evaporated to 25 cc. and the product precipitated with absolute alcohol. The precipitate was washed with acetone and ether, and dried in a vacuum desiccator. Drying at 60° in a vacuum caused slight discoloration, while at 100° considerable darkening occurred. Corresponding samples of barium ribosephosphate were prepared simultaneously from guanylic and adenylic acids. Their composition agreed with that of barium ribosephosphate.



## Ribosephosphoric Acid

*Barium Ribosephosphate from Adenylic Acid*

4 935 mg. substance: 27 180 mg. ammonium phosphomolybdate  
 70 00 " " : 42 60 " BaSO<sub>4</sub>

C<sub>5</sub>H<sub>9</sub>O<sub>8</sub>PBa. Calculated. P 8 46, Ba 37 6  
 Found. " 7 99, " 35 81

*Barium Ribosephosphate from Guanylic Acid*

4 500 mg substance: 25 360 mg. ammonium phosphomolybdate  
 60 00 " " : 36 80 " BaSO<sub>4</sub>

C<sub>5</sub>H<sub>9</sub>O<sub>8</sub>PBa. Calculated. P 8 46, Ba 37 6  
 Found. " 8 19, " 36 09

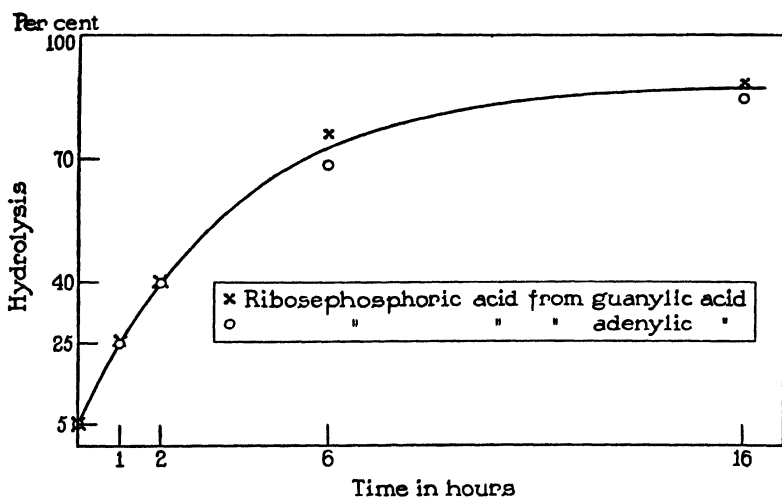


FIG 1. The rates of hydrolysis of the ribosephosphoric acids from guanylic and adenylic acids respectively, in 0.01 N HCl at 100°.

The rotations of these two substances were taken first, as sodium salts, and secondly, when diluted with an equal volume of saturated borax solution. The sodium salts in solution were prepared by exactly removing barium with the calculated quantity of sodium carbonate.

*Sodium Ribosephosphate from Guanylic Acid*

$$[\alpha]_D^{25} = \frac{-0.73^\circ \times 100}{3.750 \times 2} = -9.73^\circ \text{ (in water)}$$

$$[\alpha]_D^{25} = \frac{+1.46^\circ \times 100}{3.750/2 \times 2} = +38.9^\circ \text{ (diluted 1:1 with saturated borax solution)}$$

*Sodium Ribosephosphate from Adenylic Acid*

$$[\alpha]_D^{25} = \frac{-0.72^\circ \times 100}{3.750 \times 2} = -9.60^\circ \text{ (in water)}$$

$$[\alpha]_D^{25} = \frac{+1.33^\circ \times 100}{3.750/2 \times 2} = +35.5^\circ \text{ (diluted 1:1 with saturated borax solution)}$$

These results indicated the identity of the ribosephosphoric acids from guanylic and adenylic acids. Further confirmation of this conclusion was obtained by comparison of the rates of hydrolysis of the phosphoric acid residue.

Fig. 1 shows that they hydrolyze at the same rate.



# THE MELTING POINT OF NATURALLY OCCURRING *n*-NONACOSANE

## A CORRECTION

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In a recent paper, "Further studies on the wax-like coating of apples," Markley, Hendricks, and Sando (1) reported the melting point of *n*-nonacosane from Rome Beauty apple cuticle as 65.1° and from Ben Davis cuticle as 64.7°. A subsequent investigation of our method and apparatus for determining melting points led to the discovery that melting temperatures of 1–1.5° too high were being observed. The increase in melting temperatures was apparently due to inadequate thermal circulation resulting from an insufficient quantity of liquid in the outer jacket of the modified Thiele tube. The melting point of *n*-nonacosane taken in the same apparatus after addition of more sulfuric acid was found to be 63.4–63.7° and in a large capacity (800 ml.) oil bath, stirred at high speed, as 63.5–63.7°, which data agree with those previously observed by Sando (2) and by Chibnall and co-workers (3).

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## THE PREPARATION AND PROPERTIES OF THYRO- GLOBULIN\*

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Current procedure for the preparation of thyroglobulin is, in general, that of Oswald (1), involving extraction of the thyroid glands with 0.9 per cent saline and precipitation of the thyroglobulin from the extract either by half saturation with ammonium sulfate, or by means of acetic acid, followed by one or more repetitions of either process. Both methods gave Oswald products with identical properties, although he appears to have preferred the use of ammonium sulfate. According to Oswald, the nucleoprotein which had been recognized by Gourlay, Hutchinson, and others to be present in the extracts (for a review of the earlier literature see Oswald) is precipitated between 50 and 100 per cent saturation with ammonium sulfate. Although the product so obtained had a content of but 0.16 per cent of phosphorus, this statement appears to have been accepted by subsequent workers.

It is shown in the present communication that extracts of hog thyroids, when brought to pH 4.8 to 5.0 in the cold, deposit a product with the properties of a nucleoprotein. After its removal the thyroglobulin may be precipitated from the neutralized solution by half saturation with sodium sulfate at 35° or with ammonium sulfate. The use of acetic acid as a precipitant for the thyroglobulin (*cf.* Barnes (2)) cannot be recommended if undenatured thyroglobulin is desired, since the protein is extraordinarily sensitive to acidification. It is appreciably denatured by even a single brief precipitation with acetic acid in the cold, whereas after removal of the impurity mentioned above, it may be precipitated

\* The work described in this communication was carried out in part under the Harkness Research Fund of the Presbyterian Hospital.

many times by sulfate without denaturation. The contaminating protein, on the other hand, is stable to acetic acid, and, in common with other nucleoproteins, is precipitated, as is thyroglobulin, at half saturation with sulfate. It is, however, partly denatured in the process. It is present in far smaller amount in hog glands than is thyroglobulin itself, and is also apparently a contaminant of thyroglobulin prepared by the ordinary method from sheep, beef, and human glands.

#### EXPERIMENTAL

*Separation and Isolation of Thyroid Proteins*—Thyroids, as taken directly from hogs at the slaughter-house, were dropped into ice water, washed several times with ice water, and let stand overnight under ice water to remove blood. The glands were then trimmed, kept in the cold except during the manipulations, cut up, and run rapidly through a meat chopper. The weight of pulp in this case was 850 gm. The iodine content was 0.03 per cent, or 255 mg. The pulp was pressed out as thoroughly as possible in a hand-press and the extract was immediately chilled. The cake was stirred with 300 cc. of chilled 1 per cent sodium acetate solution (to provide a small amount of electrolyte), ground rapidly in mortars, and again squeezed in the press. The 850 cc. of press-juices, containing 234 mg. of iodine, or 92 per cent of the amount in the pulp, were diluted to 1 liter, stirred thoroughly with about 100 cc. of toluene to assist in removal of fat, let stand overnight, and whirled in a refrigerating centrifuge.<sup>1</sup> As much toluene and cream as possible was sucked off, and the residual solution was poured from the sediment and found to contain 216 mg. of iodine. 50 per cent acetic acid was then cautiously added until the precipitate first formed seemed at its maximum. The pH was 5.0 (bicolor standard method (3), bromocresol green). The solid was centrifuged off (Fraction A). The 850 cc. of supernatant liquid, containing 185 mg. of iodine, were neutralized to litmus, diluted to 2 liters, and precipitated with 2 liters of saturated sodium sulfate solution at 35°, sodium sulfate being used instead of ammonium sulfate in order to avoid the necessity of dialysis before nitrogen could be determined. Precipitation commenced when 1100 cc.

<sup>1</sup> Made by the International Equipment Company, Boston. This instrument was used throughout, unless otherwise stated.

of the sulfate solution had been added. The mixture was run through a Sharples supercentrifuge and the precipitate of thyroglobulin collected (Fraction B). 900 gm. of solid anhydrous sodium sulfate were added to the supernatant liquid, giving rise to a further precipitate which was again collected in the Sharples centrifuge. Since this fraction contained only 1.8 mg. of nitrogen and 0.017 mg. of iodine, it was discarded. The 3.75 liters of concentrated sodium sulfate solution remaining contained 237 mg. of thyroglobulin as calculated from the 0.038 mg. of iodine in the heat-coagulable protein obtained from 100 cc.

The thyroglobulin fraction, Fraction B, was dissolved in ice-cold water, centrifuged from small amounts of insoluble material, diluted to 1.5 liters, and precipitated with 1.5 liters of saturated sodium sulfate solution at 35°. The mixture was run through the Sharples centrifuge, resulting in a loss of 6.1 mg. of iodine, calculated as 1.02 gm. of thyroglobulin, in the effluent. The precipitate was redissolved in 1.5 liters of water, and an attempt was made to fractionate it by means of sodium sulfate. A slight turbidity developed in the solution when 700 cc. of warm saturated sodium sulfate had been added. The mixture was centrifuged at room temperature, and since only traces of brownish material had deposited, this was discarded and 200 cc. more of sodium sulfate solution were added to the supernatant liquid, bringing the concentration of added sulfate to 38 per cent of saturation. At this point only 5.4 per cent of the total yield was precipitated (Fraction B<sub>1</sub>). The addition of only 100 cc. more of sodium sulfate solution (42 per cent saturation) brought down 80 per cent of the thyroglobulin (Fraction B<sub>2</sub>), while the addition of 700 cc. more to the supernatant liquid from this yielded an additional 14.6 per cent (Fraction B<sub>3</sub>). The final solution contained nitrogen equivalent to 0.48 gm. of thyroglobulin. The three fractions were filtered through small amounts of paper pulp and Fraction B<sub>2</sub> was run through a Berkefeld filter in addition. As will be seen from Table I, Fractions 13-B<sub>1</sub>, 13-B<sub>2</sub>, and 13-B<sub>3</sub> were practically identical in optical rotation and iodine content, and were almost phosphorus-free. Thus no fractionation of the crude Fraction B had been effected, except that Fraction B<sub>1</sub> contained somewhat more color than the other fractions. The yields of the three fractions calculated on the basis of 15.8 per cent of nitrogen, were 1.2 gm., 17.5



gm., and 3.1 gm., respectively, or a total of 21.8 gm. Since the original press-juice contained 0.234 gm. of iodine, and the thyroglobulin recovered contained 0.6 per cent of iodine, the yield should have been  $0.234 \div 0.006$ , or 39 gm. The actual yield was therefore 56 per cent of the theoretical on the assumption that all of the iodine in the press-juice was thyroglobulin iodine. The yield would be improved by recovery of the thyroglobulin in the portions discarded in the present work, especially from the supernatant liquids from the first two reprecipitations of Fraction A (below).

TABLE I  
*Thyroglobulin Preparations*

Preparation and fraction No	Source	Precipitation at pH 4.8 to 5.0	$[\alpha]_D$	Iodine	Phosphorus	Nitrogen
			degrees	per cent	per cent	per cent
13-A	Hog	+	-48	0 21	0 44	15 8
13-B <sub>1</sub>		-	-57	0 59	0 05	
13-B <sub>2</sub>		-	-58	0 57	0 02	
13-B <sub>2</sub> , dialyzed		-	-59	0 58		
13-B <sub>3</sub>		-	-56	0 58	0 02	
12-A	Hog	+	-33	0 31	0 33	15 5
12-F		-	-61	0 57		
12-F, dialyzed		-	-59	0 56		
9-A <sub>1</sub>	Bovine	+	-46		0 6	
9-A <sub>2</sub>		+	-61		0 04	

All values calculated to the ash-free basis. Iodine determinations according to Leland and Foster (4); phosphorus by the Pregl-Lieb method (5).

Fraction A, containing the impurity usually precipitated with the thyroglobulin, was taken up in 50 cc. of 2.5 per cent sodium acetate solution, diluted with water to 500 cc., neutralized to litmus with N sodium hydroxide, mixed thoroughly with toluene, and centrifuged. The aqueous layer was again acidified to pH 5.0 and the mixture was centrifuged, Fraction A being precipitated. The supernatant liquid in the case of Preparation 13 contained 13.3 mg. of iodine or 2.2 gm. of thyroglobulin, much of which could be recovered. After a second reprecipitation of Fraction A the supernatant liquid contained only 1.4 mg. of iodine. Fraction A was redissolved as before, centrifuged, and run through

an 8 inch Berkefeld V filter, the reddish, slimy filter residue being washed with 250 cc. of 0.5 per cent sodium acetate solution which had been neutralized to litmus. The clear brownish red filtrate was acidified as before, the precipitate was redissolved, and the solution was diluted to 225 cc. and precipitated with an equal volume of warm, saturated sodium sulfate solution. Although this freed the fraction from much of the reddish impurities, the process resulted in extensive denaturation, so that the properties of the fraction recorded in Table I under Fraction 13-A were obtained with the small portion which could be redissolved in neutral sodium acetate solution.

The principal thyroglobulin fractions showed no analytical differences before or after dialysis in collodion bags, nor was there any loss of protein (Fractions 12-F, 13-B<sub>2</sub>, Table I). Nor were analytical differences detectable on the ash-free basis between portions of thyroglobulin precipitated by alcohol or by coagulation from a sodium sulfate solution at 85–90° for 15 minutes. After being washed with redistilled alcohol and acetone the protein readily attained constant weight in a high vacuum over phosphorus pentoxide at room temperature, and lost little or no additional weight at 65°. The ash-free nitrogen content of preparations precipitated and dried as above varied between 15.5 and 15.8 per cent, in better agreement with the values given by Blum (6) than with the higher figures of Oswald.

Numerous attempts were made to effect a fractionation of thyroglobulin by partial precipitation with sodium sulfate, but with no positive results as far as could be judged from the optical rotation and the nitrogen and iodine content.

*Precipitation of Thyroglobulin by Acetic Acid*—The great sensitivity of thyroglobulin to acetic acid, even in the cold, is shown by the following experiment.

25 cc. of a 0.6 per cent solution of Fraction 13-B<sub>1</sub> were diluted with 25 cc. of water, chilled in ice water, and acidified with acetic acid. A portion of the heavily opalescent mixture was centrifuged for 1 hour in the cold (precipitate, Fraction a, supernatant liquid, pH 4.1),<sup>2</sup> while the remainder was allowed to stand at room

<sup>2</sup> After the solutions had been warmed to room temperature and centrifuged, these determinations, with a glass electrode, were kindly made by Mr. F. Rosebury of the Department of Biological Chemistry.

temperature for 1.5 hours before being centrifuged (precipitate, Fraction b, supernatant liquid, pH 4.0).<sup>2</sup> Fractions a and b were then redissolved in water with the aid of enough *N* sodium hydroxide solution to insure neutrality to litmus. Fraction b was strongly opalescent, but could be cleared by prolonged centrifuging in the cold. Approximately equal amounts of Fractions 13-B<sub>1</sub>, a, and b were then added to excess 0.1 *M* acetate buffer at pH 4.8 and 4.6 with the following result, in which  $\pm$  to +++++ indicates slight opalescence to complete precipitation.

Fraction pH	13-B <sub>1</sub>		a		b	
	4.8	4.6	4.8	4.6	4.8	4.6
0.5 hr. in ice water	—	—	+	++	+++	++++±
18 hrs. in ice box	±	++	+++++	+++++	++++±	+++++

The main fraction, Fraction 13-B<sub>2</sub>, behaved in the same way.

Thus exposure of thyroglobulin to a pH of 4.1 in the cold for as little as 1 hour results in partial denaturation, and the process is greatly accelerated at room temperature. Even at pH 4.6 to 4.8 appreciable denaturation occurs in 18 hours in the cold. The isoelectric range, or rather range of minimum solubility of thyroglobulin which has not previously been treated with acid, appears to be between pH 3.8 and 4.3.<sup>2</sup> Thus thyroglobulin which has been partially denatured by acid becomes precipitable at hydrogen ion concentrations at which native thyroglobulin remains in solution. The shift in the precipitation range is in the direction of that of impurity. Fraction A, so that the two proteins would tend to remain together in the acetic acid method of isolation.

This is borne out by experiments with a dried, and therefore, largely denatured, acetic acid-precipitated bovine thyroglobulin preparation kindly placed at the writers' disposal by Dr. H. D. Dakin. 10 gm. of the protein were taken up in 100 cc. of water in the cold and treated with *N* sodium hydroxide solution until the initially acid supernatant liquid was neutral to litmus after standing overnight in the ice box. The volume was then made up to 600 cc. and additional sodium hydroxide added to make the excess alkalinity 0.02 *N*. The mixture was stirred in the cold for 1.5

hours, centrifuged, and acidified with acetic acid. The precipitate (Fraction 9-A<sub>1</sub>, see Table I) resembled the corresponding fraction from hog glands in its relatively high phosphorus content and low optical rotation, while a second extraction of the residue yielded both a low phosphorus, high rotating acetic acid-precipitable thyroglobulin (Fraction 9-A<sub>2</sub>) and a relatively small non-precipitable portion (Fraction 9-B) which came down with an equal volume of sulfate.

It was also found possible to separate dried thyroglobulin from pathological human glands into a portion precipitable by acetic acid at pH 4.8 to 5.0 in the cold, and a portion which was non-precipitable. Aqueous extracts of fresh sheep thyroids also contained a fraction insoluble at pH 4.8 to 5.0 in the cold.

Chemical and physiological studies on the fractions described in this communication are being continued.

The writers are greatly indebted to Ethel Benedict Gutman for many of the iodine analyses.

#### SUMMARY

Aqueous extracts of hog thyroid glands contain a fraction precipitable in the cold at pH 4.8 to 5.0. This fraction has the properties of a nucleoprotein and is a contaminant of thyroglobulin isolated by the classical methods of Oswald. Its partial denaturation by half saturated sulfate would tend to eliminate a portion of it in repeated sulfate precipitations of thyroglobulin, but the rapid alteration undergone by thyroglobulin in the presence of acetic acid tends to make mixtures of thyroglobulin and the contaminant inseparable. If entirely undenatured thyroglobulin is desired, exposure to acetic acid at a pH below 4.8, even in the cold, should be avoided.

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## KETONE SUBSTANCE PRODUCTION AND DESTRUCTION IN CERTAIN TISSUES OF DIABETIC DOGS\*

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The metabolism of the acetone substances in the intact organism has been the subject of numerous investigations, but researches on the function of individual organs have been relatively meager. Embden and his collaborators (1, 2) concluded from experiments on excised tissue perfused with the animal's own blood that the liver was the source of acetone bodies. Chaikoff and Soskin (3) showed that injected acetoacetic acid remained longer in the blood of depancreatized dogs than in normal dogs, despite the fact that the muscles of the two types of experimental animals utilized the ketone acids with equal facility. They concluded that the liver of the depancreatized animal produced acetone substances.

In a previous paper (4) the function of the liver, striated muscle, and the gastrointestinal tract was investigated by means of analyses of afferent and efferent blood samples for total acetone substances. The liver was found to be the most constant source of acetone substances, regardless of the action of the other organs. Blood samples drawn simultaneously from striated muscle and the gastrointestinal tract showed that these organs had the same qualitative effect on the blood concentration of total acetone substances in most experiments. In the present work, the observations were extended to include the heart, brain, and testicle.

### *Methods*

Experimental diabetes was induced in eighteen dogs after a preliminary fast which lasted from 24 to 72 hours. Ten animals

\* The expenses of this research were met in part by a grant from the Research Fund of the Yale University School of Medicine.

were phlorhizinized (5) and eight were depancreatized (6). Blood samples were drawn from the phlorhizinized animals after definite amounts of acetone substances appeared in the urine, and from the depancreatized dogs 2 to 5 days after operation. The animals were anesthetized with amytal.

Since the concentration of acetone substances is the same throughout the arterial system, the femoral artery was used as a convenient source of afferent blood for all the organs. The efferent blood samples of the striated muscles and heart were obtained from the femoral vein and coronary sinus respectively. Observations on the brain were made by means of a window trephined in the cranium which exposed the superior longitudinal sinus, from which the brain efferent blood was sampled. The venous blood of the testicle was drawn from the left spermatic vein caudal to the point at which it empties into the left renal vein. In every case a separate arterial blood sample was taken at the same time as the venous sample.

The method of Van Slyke and Fitz (7) with their modification (8) was employed for the analysis of total acetone substances of the blood. The error of a single determination was  $\pm 0.8$  mg. per cent.

### *Results*

In the course of obtaining data from the various organs, the femoral arterial blood of each animal was analyzed from three to seven times at varying intervals. It was thus possible to determine the variation in the concentration of acetone substances in arterial blood. Several typical experiments are presented in Table I. The fluctuations observed made it necessary to take an arterial blood sample each time a venous sample was drawn.

Differences in the concentration of acetone substances in the afferent and efferent blood which exceeded 3 times the experimental error were taken as significant. Smaller differences were listed as "no change."

*Heart*—Afferent and efferent blood samples were obtained from the heart and striated muscle simultaneously, so that a comparison might be made between the actions of the two types of muscle tissue. This comparison is presented in Table II. The number of experiments in which the acetone balance of striated muscle

was negative, zero, or positive, is presented in Column 2. The effect of heart muscle in each set of experiments listed in Column 2 is noted in Columns 3 to 5. In the eight observations in which striated muscle had a positive balance, that of the heart was positive six times, negative once, and zero once. Striated muscle was found to add acetone to the blood in two experiments, and

TABLE I  
*Variations of Acetone Concentration in Arterial Blood*

Experiment 34		Experiment 33		Experiment 31		Experiment 27	
Time	Acetone	Time	Acetone	Time	Acetone	Time	Acetone
<i>min</i>	<i>mg per cent</i>	<i>min</i>	<i>mg per cent</i>	<i>min</i>	<i>mg. per cent</i>	<i>hrs</i>	<i>mg per cent</i>
0	37	0	49	0	24	0	11
15	14	20	31	41	29	5	9
30	13	45	27	90	6	7	5
40	5	60	45	105	22	11	10
55	9	75	21	450	49	11 5	5
		90	26	460	30		

TABLE II  
*Correlation of Changes of Blood Acetone Produced by Muscle with Those Produced by Heart*

Muscle		Heart		
(1)	(2)	Added (3)	No change (4)	Removed (5)
Added	2	2	0	0
No change	5	0	3	2
Removed	8	1	1	6
Total	15	3	4	8

The readings are for the number of experiments in which acetone was added to, remained unchanged, or was removed from the blood.

simultaneously the heart also was found to add acetone twice. The arteriovenous difference of striated muscle was within 3 times the experimental error in five cases, while the heart removed acetone from the blood twice, and made no change three times. Thus, in eleven of fifteen determinations, cardiac and striated muscle exhibited the same acetone balance.



*Brain*—Ten observations were made on the brain in six depancreatized dogs, and thirteen afferent and efferent blood samples were obtained from five phlorhizinized animals. Table III contains the results of these experiments in summary. In eighteen of twenty-three cases, no change occurred in the concentration of ketones in the blood passing through the brain. In the five experiments in which the difference was greater than 3 times the

TABLE III  
*Summary of Effect of Brain on Blood Acetone*

Type of diabetes	Added	No change	Removed
Phlorhizin	0	11	2
Pancreatic	2	7	1
Total	2	18	3

The readings are for the number of experiments in which acetone was added to, remained unchanged, or was removed from the blood.

TABLE IV  
*Correlation of Changes of Blood Acetone Produced by Muscle with Those Produced by Testicle*

Muscle		Testicle		
(1)	(2)	Added (3)	No change (4)	Removed (5)
Added		1	0	4
No change		0	5	1
Removed		0	3	1
Total	15	1	8	6

The readings are for the number of experiments in which acetone was added to, remained unchanged, or was removed from the blood.

experimental error, the acetone balance was positive three times and negative twice. No simultaneous observations were taken on muscle, since in most cases, the brain had no effect on the concentration of acetone substances in the blood.

*Testicle*—It seemed desirable to compare the effect of the testicle and striated muscle on blood acetone, since a similarity between the actions of striated muscle and the gastrointestinal tract had

been noted (4). Blood was drawn from the femoral artery, femoral vein, and spermatic vein and the effect of the two organs was compared. This correlation is presented in Table IV. In the five experiments in which the acetone balance of striated muscle was positive, the testicle added acetone once and removed it four times. Muscle made no change six times, while the testicle made no change five times and removed acetone once. Of the four experiments in which muscle removed acetone, testicle removed it once and made no change in three of these experiments. In only one case of a total of fifteen did the testicle cause an increase in the ketone concentration of the blood passing through it.

The observations were usually made on two or three organs simultaneously, which made it possible to calculate the coefficients of correlation of the actions of the various viscera. The coefficients are presented in Table V. There is a significant positive

TABLE V  
*Coefficients of Correlation Among the Various Organs*

	Coefficient
Striated muscle and liver	-0 087 $\pm$ 0 107
“ “ “ testicle	-0 165 $\pm$ 0 169
“ “ “ gastrointestinal tract	0 459 $\pm$ 0 085
“ “ “ heart	0 727 $\pm$ 0 082

correlation between striated muscle and the gastrointestinal tract, and between striated muscle and cardiac muscle. The coefficients for striated muscle and liver, and striated muscle and testicle indicate that these organs act in complete independence.

#### DISCUSSION

In the present experiments a study was made of the acetone production of the various organs of the dog. It was observed that the concentration of ketones in the arterial blood was not constant, but varied within wide limits (Table I). Such variations might be due to the fact that each of the organs of the body can affect the acetone concentration of the blood. The arterial blood concentration would thus be the resultant of the changes effected by all the organs.

In a previous paper (4) it was demonstrated that the liver was

the organ of the body most prone to ketosis during deprivation of carbohydrate. This may be due in part to its large energy requirement (9) which is probably satisfied by fat in the diabetic organism, and by fat and glucose in normal individuals. The fat may be oxidized to the 4-carbon stage without the simultaneous catabolism of carbohydrate. In diabetes, because less glucose is oxidized, the liver cannot completely catabolize the acetone substances, which therefore diffuse out into the venous blood.

The brain was in acetone equilibrium eighteen out of the twenty-three times it was tested. The respiratory quotient of unity found for the brain (10) might result from the oxidation of glucose, acetoacetic acid, lactic acid, or a combination of the above three substances. There is considerable evidence to show that the cerebral cortex does not oxidize glucose either in the normal or diabetic organism (10, 11). In the present experiments we have been unable to demonstrate the removal of acetone substances by the brain. Thus, it may be said that the brain oxidizes mainly lactic acid, and that the amounts of acetoacetic acid it may oxidize are too small to be detectable with our method.

The character of the food mixture oxidized by the testicle was investigated by Krebs (12). The evidence indicates that this organ, like the cerebral cortex, can oxidize carbohydrate only after its conversion to lactic acid. Unlike the cerebral cortex, however, the testicle can oxidize fat (13). The food mixture oxidized by the testicle in diabetes also consists of fat and lactic acid, since insulin is probably not required for the oxidation of lactic acid (10). Satta (14) has shown that lactic acid is an antiketogenic substance. Therefore the acetone balance of the testicle would be determined by the proportions of the two foodstuffs oxidized, fat and lactic acid. In those cases in which the antiketogenic value of the lactic acid metabolized exceeded the ketogenic value of the fat, the testicle would remove acetone substances from the blood and oxidize them to carbon dioxide and water. In studying the acetone balance of the testicle, either a positive or a zero balance was observed in fourteen of fifteen observations. In those experiments in which there was a positive balance, it is probable that there was more than enough lactic acid oxidized to permit the complete oxidation of the fat used by the testicle. This excess permitted the removal and the oxidation of acetone substances from the blood.

The actions of the gastrointestinal tract, which consists in part of smooth muscle, and striated and cardiac muscle in these experiments showed a high degree of correlation (Table V). These three organ systems have therefore been grouped together in the following discussion. It is possible that the acetone substances which are apparently removed or discharged at various times might depend on the storage of the ketones at one time, and a return to the blood stream at a later time. However, it is also possible that the ketone acids which were removed were oxidized; and those cases in which the organs added acetone substances to the blood might be due to the incomplete oxidation of the fatty acids. There is some evidence in favor of the latter possibility. Chaikoff and Soskin (3) have demonstrated that the muscles of depancreatized dogs can oxidize acetoacetic acid. Sweet and Quick (15) found that depancreatized dogs oxidized butyric acid. Our own experiments indicate that the liver added 12 to 100 gm. of acetone substances per day to the blood, and only a small fraction was eliminated through the kidneys and lungs. Thus, it is not improbable that the acetone substances removed from the blood by muscle were oxidized, despite the fact that presumably no glucose was oxidized (16, 17). As in the case of the testicle, so with muscle, the oxidation of ketone acids may be due to the oxidation of lactic acid (18). In this regard, it is interesting to recall that the liver, the only organ which cannot split glucose to lactic acid (19), is likewise the only organ which is consistently ketogenic.

#### SUMMARY

1 The influence of various organs on the concentration of acetone substances in the blood passing through them was studied in eight depancreatized and ten phlorhizinized dogs. In eight of fifteen experiments the heart removed acetone substances from the blood and on three other occasions added ketone acids to the blood. The brain made no change in the ketone acids in eighteen of twenty-three experiments. The testicle removed acetone from the blood six times, and made no change eight times in fifteen experiments.

2 These results give some indication of the character of the foodstuffs oxidized by the individual organs. Thus, only organs which oxidize fat and carbohydrate, the latter in the form of glucose, are capable of producing acetone substances in diabetes.

This applies to skeletal muscle, gastrointestinal tract, cardiac muscle, and the liver. The testicle oxidizes fat and lactic acid. Lactic acid is ketolytic and therefore the testicle frequently removes acetone from the blood. Brain cortex is unique in that it does not oxidize fat, and has no demonstrable effect on the acetone concentration of the blood.

3. The observations were made on two or more organs simultaneously, so that it was possible to correlate the effects obtained. The testicle and liver each had effects independent of those of striated muscle. Striated muscle, heart, and the gastrointestinal tract, on the other hand, exhibited a high degree of correlation, indicating a similarity in the metabolic processes of these tissues. In approximately one-third of the experiments muscle was removing acetone substances despite the constant addition of these substances by the liver.

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## GLYCINE SYNTHESIS IN PSEUDOHYPERTROPHIC MUSCULAR DYSTROPHY

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In studies of the conjugation of benzoic acid in man, Quick (1) considers from his data that the body has no store of preformed glycine and that benzoic acid acts as a stimulus for the synthesis of this amino acid, glycine production increasing with increased amounts of benzoic acid up to a certain maximum. By feeding sodium benzoate he determined the rate of glycine formation and the maximum production per unit of time which he found to be 0.009 gm. per kilo per hour. Recent reports of the favorable effect of glycine administration in cases of muscular dystrophy (2) caused us to feel that a study of the production of glycine in response to sodium benzoate administration might be instructive in such cases. In connection with some other work, an opportunity was afforded for carrying out such a study.

### EXPERIMENTAL

Three boys, well advanced cases of pseudohypertrophic muscular dystrophy,<sup>1</sup> were studied along with three orthopedic cases closely corresponding in age and weight. These controls were of normal musculature and in no way dystrophic. All of these subjects had been on diets of low glycine content for a period of about 6 weeks before the experiment.

<sup>1</sup> These cases were diagnosed by Dr. J. A. Key, Professor of Orthopedic Surgery, Washington University Medical School, and Drs. L. C. Abbott and C. H. Crego, Jr., of the Shriners' Hospital for Crippled Children in St. Louis. The patients have all shown symptoms of muscular weakness from birth, progressively becoming worse. Two of the patients have been unable to walk or rise to the standing position since entering the hospital last summer. They are able to slide in the sitting position. The other patient can walk but not arise from the sitting position.

The subjects were fed a light breakfast of pudding prepared from starch, 32 per cent cream, and sugar at 7.30 a.m. This meal furnished little glycine and prevented the gastric discomfort sometimes experienced when sodium benzoate is taken on an empty stomach. At 8.00 a.m. 0.1 gm. of benzoic acid (as benzoate) per kilo of body weight was given in 125 cc. of water. This dos-

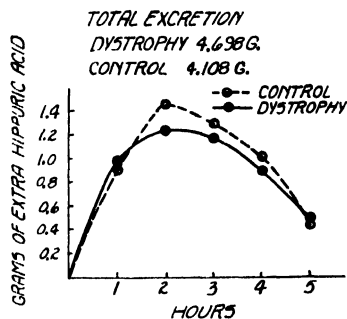


CHART 1

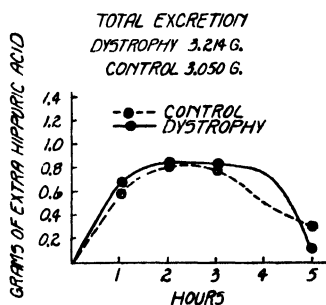


CHART 2

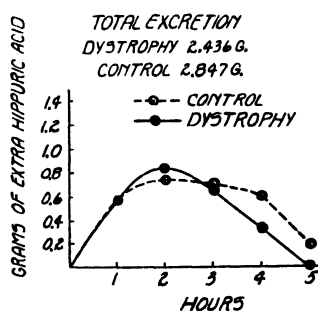


CHART 3

CHARTS 1 TO 3. Rate of excretion of hippuric acid

age was well tolerated. Hourly urine samples were collected, 75 cc. of water being given each hour. Hippuric acid was determined according to the method described by Griffith (3).

### Results

The hourly excretion of hippuric acid (Charts 1 to 3) shows a very close correlation between each dystrophy case and his con-

trol as do the total amounts excreted during the 5 hour period. The maximum glycine production as calculated from the hour of greatest hippuric acid excretion (Table I) likewise showed no significant differences between the dystrophy cases and controls.

From these experiments it seems that in pseudohypertrophic muscular dystrophy the capacity of the organism to produce glycine in response to benzoic acid differs in no way from that of the normal individual.

TABLE I  
*Maximum Glycine Production*

	Age	Weight	Glycine per kilo per hr
	<i>yr</i>	<i>kg</i>	<i>gm</i>
Dystrophy	11	34 6	0 015
Control	12	36 0	0 017
Dystrophy	9	28 6	0 012
Control	8	27 2	0 012
Dystrophy	8	21 6	0 016
Control	8	22 5	0 014

The writers are indebted to the staff of the Shriners' Hospital for Crippled Children at St. Louis, for making the children available for study and to Miss Alice Sauer in particular for her excellent cooperation.

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# THE RATE OF ABSORPTION OF GLUCOSE FROM THE INTESTINAL TRACT

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Using an obvious but none the less ingenious method, Cori (1) examined the rate of absorption of glucose and other sugars from the intestinal tract and came to the conclusion that the absorption of hexoses is independent of the absolute amount and concentration of sugar in the intestine, giving a straight line relationship between the absorption rate and time. In a study of the rate of absorption of glucose in relation to the diet stated in a previous paper (2) in which the method devised by Cori was used we found no such straight line absorption but instead a marked falling off in the absorption rate during the successive hours after glucose administration. Pierce, Osgood, and Polansky (3) obtained results which led them to conclude that the percentage of glucose absorbed appeared to be dependent upon the amount of glucose remaining unabsorbed in the alimentary tract. Cori *et al.* (4) explain the findings of these investigators upon the basis that sufficient glucose was not fed to allow the absorption rate to continue at the initial level. Burget, Moore, and Lloyd (5) using Cori's method found that the rate at which glucose is absorbed decreases with time, a finding in agreement with their results with the isolated ileum loop in the dog. It is unfortunate that their results for the rat are not presented in more detail. The failure of subsequent investigators to confirm the conclusions reached by Cori (1) have made it desirable to reexamine the absorption of glucose from the intestinal tract.

## *Methods*

The method used was essentially the same as that of Cori (1). A known amount of glucose solution was introduced into the

stomach of each of a reasonably homogeneous group of fasted albino rats and at various periods thereafter groups of the rats were killed and the amount of glucose remaining in the gastrointestinal tract determined. This, then, gave the average rate of absorption for the group. Since it is a group figure which is being obtained, it is essential that the group be as homogeneous as possible. In a given experiment all of the rats were of the same sex and born within a period of 20 days. The body weight distribution was made as even as possible throughout the group. In certain other details our procedure was slightly different from that used by Cori. Our rats were fasted but allowed water for 40 hours. We chose to give each rat a definite dose of glucose in relation to its body size. To do this a No. 8 or No. 10 urethral catheter of soft rubber was attached to a 5 ml. burette graduated in 0.02 ml. intervals. At the top of the burette a small Lucr syringe was inserted in a rubber stopper which closed the burette and served to apply air pressure to the solution. The burette and catheter were filled with the sugar solution, care being taken to exclude air bubbles from the catheter tubing, the tip of the catheter was introduced into the stomach of the rat as described by Cori, and a given amount of sugar solution, as measured by the burette, delivered. We attained a very good accuracy of sugar administration in this manner for variable volumes. When the catheter was withdrawn, it was still filled with solution to the tip. We can confirm Cori in finding that a negligible amount of sugar solution adheres to the outside of the tip. In other respects Cori's technique was followed. The gastrointestinal tract was removed and treated in the same manner. Glucose was determined by the Somogyi (6) modification of the Shaffer-Hartmann method.

*Body Size*—Cori has assumed that the absorbing surface of the intestine is proportional to the body weight. His data are hardly sufficient for examining this point, for the range of body weights which he examined was only 100 to 180 gm. He chose as his absorption coefficient the amount of substance absorbed per 100 gm. of body weight in 1 hour. Elsewhere (2) we have used as the *absorption coefficient* the amount of substance absorbed per 100 sq.cm. of body surface per hour on the basis that it was most reasonable to assume proportionality between the absorbing surface of the intestine and the body surface area (and hence the

maintenance metabolism) and because we had found that lethal or effective doses of various substances generally bore a more constant relation to body surface than to body weight. Pierce, Os-good, and Polansky (3) found a slightly closer relationship between the absorption of glucose and body surface than between its absorption and body weight. Here again the distribution of body weights does not cover a range adequate to give a basis for any general conclusions.

To determine the best measure of body size upon which to base the *absorption coefficient* for glucose, a group of male and female rats of widely varying age and size was given 0.8 cc. (456 mg.) per sq.dm. of body surface of a 57 per cent glucose solution after a fasting period of 40 hours and killed in 1 hour. The data comprise Table I. There is no question but that body surface is the measure of choice as a basis for intestinal absorption. The coefficient of variation of the hourly rate of glucose absorption is smaller when expressed per sq.dm. of body surface than when expressed per 100 gm. of body weight. The coefficients are 18.4 on the surface basis and .265 on the weight basis. Obviously, determinations of intestinal absorption rates are better equalized for differences in the size of the rats by referring them to surface rather than weight.

Harris and Benedict (7) in commenting on the same relation between the variability of their basal determinations referred to weight and surface hesitated to ascribe to this relation any significance as a criterion of the relative merits of the two methods of expressing the intensity of basal metabolism. They point out that, from mathematical considerations only, one would expect the surface area of animals of a given species to possess a smaller percentage variability than the body weight. Hence, they argue, the determined basal heat production divided by the body surface will give a less variable series of ratios than will the basal heat production divided by the body weight, and the relation becomes thus a "mathematical necessity" with no physiological significance. A similar view could be taken of the relation of various measurements, such as kidney weight (8) and in the present case glucose absorption from the intestine to body surface.

The argument of Mitchell and Carman (9) against the view of Harris and Benedict with a slight substitution of words might be well used here: "The argument of Harris and Benedict fails to

TABLE I

**Comparison of Absorption Coefficients (Mg per Hour) for Glucose in Relation to Body Weight and Body Surface after a Dose of 456 Mg of Glucose per Sq Dm of Body Surface in 57 Per Cent Concentration**

[illegible]

consider one important fact, namely, that the variability of the *ratios* of [the rate of glucose absorption] to either weight or surface depends not only upon the variability of the original [glucose absorption] determinations and of the body surfaces and body weights, but also upon the correlations existing between . . . the [glucose absorption] and the surface. In fact, since [glucose absorption] is so largely determined by size of body, the degree of correlation is the determining factor in the matter. Therefore, the smaller variability of [glucose absorption] per [sq.dm.] of body surface may be fairly interpreted to mean a greater correlation between the two than between [glucose absorption] and the body weight. This greater correlation, may be taken to mean a closer causal relation, certainly of physiological significance."

Henceforth is to be understood as the *absorption coefficient* the amount of the substance under consideration absorbed per unit of time in relation to body surface; *e.g.*, mg. per hour per 100 sq.cm. of body surface in the case of the albino rat. For the determination of body surface we have used the formula of Carman and Mitchell (10). The average absorption coefficient for the rats in Table I receiving 456 mg. of glucose per sq.dm. is 110 mg. per hour. For the females alone it is 109 mg. and the males, 113 mg., an insignificant difference. Recalculating Cori's figures (1), we find an average for eight rats of 92 mg. This was with variable doses averaging 301 mg. per sq.dm. of body surface. With a dose of 228 mg. of glucose (Table II) we found an absorption coefficient of 90.

In connection with our conclusions in regard to body surface and the absorption coefficient it could be argued that our variability was less in relation to body surface because our dose of glucose was proportional to body surface. An indirect argument opposed to this idea is the observation of Orr-Ewing (11) that consistent glucose tolerance tests are obtained in animals of widely different weight only if the dose is adjusted to the body surface.

*Rate of Absorption*—Thirty male rats were each given 0.4 cc. of a 57.02 per cent glucose solution per 100 sq.cm. of body surface and six of these were killed every hour thereafter for 5 hours. This dose of 228 mg. of glucose per sq.dm. of body surface gave (Table II) a gradually falling rate of absorption. At the initial rate of absorption (90 mg. per hour per sq.dm.) sufficient glucose

TABLE II

Absorption period	Average body surface	Glucose absorbed per 100 sq cm		Average body surface	Glucose absorbed per 100 sq cm		Average body surface	Glucose absorbed per 100 sq cm	
		Absorption period	Average per hr		Absorption period	Average per hr		Absorption period	Average per hr

Experiment 1. Data for absorption coefficients of male rats after different amounts of glucose in a concentration of 57.02 per cent Dose of glucose, mg. per 100 sq cm. body surface. Each figure unless noted is an average of six rats

Dose, mg	Experiment 1-A 228			Experiment 1-B 456			Experiment 1-C* 684		
hrs	sq cm	mg	mg	sq cm	mg	mg	sq cm	mg	mg
1	419	90	90	419	101	101	419	138	138
2	419	168	84	419	156	78	425	182	91
3	419	146	73	419	234	78	421	249	83
4	419	228		419	296	74	432	292	73
5	419	230		418	365	73	418	390	78

Experiment 2. Effect of repeated administration of glucose. Male rats after a dose of 171 mg of glucose per sq dm. body surface and 57 mg per hr thereafter. Four rats in each group

1	493	81	81						
2	491	132	66						
3	495	231	77						
4	495	316	79						
5	496	350	70						

Experiment 3. Data for absorption coefficients of female rats after glucose in different concentrations. Each figure is an average of six rats

Concentration, per cent Dose, mg	Experiment 3-A 24 75 374			Experiment 3-B 58 20 465			Experiment 3-C 73 30 442		
1	353	97	97	352	109	109	352	128	128
2	352	148	74	351	168	84	351	196	98
3	352	216	72	351	261	87	351	252	84
4	351	272	68	350	300	75	350	312	78
5	353	325		351	345	69	351	385	77

\* Averages for four rats in each group.

was given to maintain this rate for 2.5 hours. It actually fell during the 2nd hour so that calculation of the average figure for the last hour of each period (90 mg. the 1st hour, 78 mg. the 2nd hour, 53 mg. the 3rd hour, 8 mg. the 4th hour, and 0 mg. the last hour) even indicated some absorption of glucose in the 4th hour. This calculation involves considerable error, but it is interesting that the total amount of glucose found to be absorbed over the 4 hours is 229 mg. and 228 mg. were fed.

In an attempt to prevent the falling off of the absorption rate an experiment was carried out (Table II, Experiment 2) in which the glucose in the intestine was replenished at the end of each hour. Both the initial dose of glucose and the replacement doses were half of what had been intended but the absorption period averages were better maintained than in any of the other experiments whatever the dose of sugar given. This suggests that the failure of the rate of absorption to be maintained in the preceding experiment was probably due to the decreasing quantity of glucose in the intestine.

The influence of varying the amount of glucose administered on the absorption rate has been examined in the first three experiments summarized in Table II. The highest dose caused a diarrhea in a third of the animals and they were discarded. During the 1st hour of absorption, there is a relation between the dose and the rate. After this except for the smallest dose absorption is not particularly related to the dose. Calculated for the last hour of each period (Experiment 1-B, 101, 50, 80, 58, and 76 mg. respectively; Experiment 1-C, 138, 43, 69, 44, and 99 mg. respectively) the rate is irregular and unrelated to the dose. This may be entirely due to the large error which must enter this calculation but is possibly in part due to the osmotic disturbance produced by the large quantities of hypertonic sugar solution, reaching its maximum in the rats with diarrhea and associated with the entry of water into the intestine as described by Cori (1). One part of the irregular calculated last hour absorption rates after the 1st hour on the two higher doses may have some significance, for in both experiments the absorption rate fell to its lowest level during the 2nd hour, rose again during the 3rd hour, fell the 4th hour, and rose during the 5th hour after sugar was given. To a lesser extent similar changes were found in other experiments (Table II, Experiments 3-B and 3-C).



Cori (1) found no difference in the absorption coefficient after 25, 50, and 80 per cent glucose solutions, but the dose given varied with the concentration. We have compared the relative influence of 25, 58, and 73 per cent solutions on the rate in female rats. It was impractical to give the group given the 25 per cent solution the full volume of solution, so their dose is slightly lower than the other two. All of the doses were of the same magnitude. The average figures (Table II, Experiment 3) show very clearly in the 1st hour a relation between the absorption rate and the concentration of glucose given. They both increase together. It is evident but in less degree in the coefficients of the other absorption periods. Cori found (1) that the concentration of glucose in the intestinal tract decreased steadily after a dose of strong solution. This may be the cause for the falling off in the rate of absorption even when there is an excessive amount of sugar in the intestine.

In Experiment 1-B male rats received 456 mg. per sq. dm. of body surface in 57 per cent solution and in Experiment 3-B female rats were given 465 mg. in 58.2 per cent solution. The averages give no definite evidence that sex has a significant effect upon the rate of glucose absorption under these special conditions.

#### DISCUSSION

Our results are not in agreement with those reported by Cori (1). With a 50 per cent glucose solution he found a maintained rate for 3 hours but he fed increasing amounts of glucose. In a later experiment (4) the rate was maintained over 3 hours when each group received essentially the same dose. With an 80 per cent solution, Cori found (1) that the rate of absorption of glucose had a tendency to increase during the 5 hour period. We can find no explanation for our failure to confirm these results.

We feel that the most importance should be attached to the absorption coefficient for the 1st hour. Its variability is not significantly greater than when absorption was measured over longer periods. In our hands the variation in the absorption coefficient (coefficient of variability = standard deviation of mean expressed as a percentage of the mean) for groups of twenty-two rats each of approximately the same weight and receiving doses of 442 to 684 mg. per 100 sq. cm. of body surface in each group was 14 per cent the 1st, 17 per cent the 2nd, and 12 per cent the 3rd hour. It

would seem then that the higher absorption coefficient found in the 1st hour after sugar is given is due not to any error inherent in the method and influencing particularly the 1st hour but to the fact that the rate of absorption decreases after the 1st hour.

Our experiments lead us to the conclusion that the true relation of the rate of absorption from the intestine to the amount and concentration of material in the intestine can only be established in experiments in which the stomach contents are considered separately.

#### SUMMARY

The absorption rate of glucose per unit of time from the intestinal tract of male and female rats of various ages bears a more constant relation to body surface than body weight. The amount of glucose absorbed per unit of body surface per hour of time has been used as the absorption coefficient.

The absorption coefficient is raised by increases in either the amount or concentration of glucose administered.

Whatever the dose of glucose the rate of absorption as measured by the method used here decreases with time after it is given.

*Addendum*—After this manuscript was submitted for publication, a paper by Trimble, Carey, and Maddock (12) on the rate of absorption of glucose in the dog appeared. Their results are interpreted to show that the rate of absorption is the same for each of the first 3 hours after glucose administration. This is apparently true under their special conditions, but the group in which absorption continued for 1 hour received 2.12 gm., the group in which absorption continued for 2 hours 3.21 gm., and the group in which absorption continued for 3 hours 4.26 gm. of glucose per kilo. This would make the 1st hour of the 3 hour period higher than that of the 2 hour period, which in turn would be higher than the 1 hour period. It is probable that there was a falling off in the absorption rate in both the 2 hour and 3 hour periods but that the increasing doses of glucose led to an apparently sustained average absorption rate.

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## THE OXIDATION OF HEMOCYANIN

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Hemocyanin is a copper-containing protein which occurs in the blood of a number of species of arthropods and mollusks. The extent of the reversible combination with molecular oxygen is a function of the partial pressure of oxygen, and like the formation of oxyhemoglobin is affected by changes in acidity and salt concentration. The ratio of combined molecular oxygen to copper is  $O_2:2Cu$  in all the hemocyanins studied from a variety of different bloods. The deoxygenated protein is colorless, while the oxygenated compound (oxyhemocyanin) has an intense blue color.

No evidence has hitherto been available as to whether the copper in hemocyanin was in the cupric or cuprous state. The usual oxidizing agents which oxidize the ferrous compounds, hemoglobin and oxyhemoglobin, to the ferric compound, methemoglobin, appear to be without effect on hemocyanin or oxyhemocyanin. We have now found that by the use of the two very powerful oxidizing agents, potassium molybdicyanide or potassium permanganate, it is possible to oxidize the hemocyanin (or oxyhemocyanin) of *Limulus polyphemus*. In this way two new proteins are formed in which the copper is in the cupric state. One of these, prepared from hemocyanin in the absence of oxygen, is colorless and we shall designate it as *methemocyanin*. The other, *oxymethemocyanin*, is formed when a solution of methemocyanin is shaken with air or oxygen; the deoxygenation of methemocyanin like that of hemocyanin may be brought about by diminishing the partial pressure of the oxygen above the solution. It is evident that, unlike methemoglobin, methemocyanin combines reversibly with oxygen.

The cupric compounds, methemocyanin and oxymethemocyanin, are reduced by the action of a variety of reducing agents,

but two particular reagents have proved of special value in this work. These are potassium ferrocyanide and 1,4-naphthohydroquinone, which are oxidized only very slowly by atmospheric oxygen at a pH value of 7. Because of this fact they may be used for titrating oxidizing agents in the presence of air. The end-point may be determined electrometrically in the usual type of oxidation-reduction cell, or in the case of naphthohydroquinone phenolindophenol may be used as an indicator. In a phosphate buffer of pH 7, this indophenol is reduced by naphthohydroquinone and reoxidized by methemocyanin (or oxy-methemocyanin) but only slowly by air. The titration of the cupric compound is therefore complete when the indophenol color (pink) has disappeared. In the use of potassium ferrocyanide an excess of the reducing agent is added and then the solution back titrated rapidly with molybdicyanide, the end-point being determined electrometrically. The reoxidation of the cuprous compound is sufficiently slow as compared with that of the ferrocyanide so that a satisfactory end-point may be obtained.

#### *Oxymethemocyanin*

In Table I are summarized a number of experiments in which oxyhemocyanin in a phosphate buffer (pH 7) was treated with varying quantities of dilute potassium permanganate. The color of the permanganate solution disappears rapidly; any excess is probably consumed by attacking some organic grouping in the protein molecule. As will be seen, the oxidizing power of the resulting solution is essentially independent of the amount of permanganate used, if more than 3.5 equivalents are added.

By adding sodium hydrosulfite to an oxymethemocyanin solution, the greenish blue color is discharged and on now being shaken with oxygen the blue oxyhemocyanin is formed. Colorimetric comparison of such regenerated oxyhemocyanin provides evidence as to whether or not the characteristic structure of the protein has been destroyed by this cycle of oxidation, reduction, and oxygenation reactions. In a number of experiments it was shown that no appreciable decomposition had occurred as judged by this criterion.

Oxymethemocyanin may be purified by precipitation with ammonium sulfate. The preparation of purified samples by two different methods is given below.

*Preparation of Purified Oxymethemocyanin*—The oxyhemocyanin used was obtained from the serum of *Limulus polyphemus* and purified by dialysis and precipitation at the isoelectric point

TABLE I  
Oxidizing Power of Crude Oxymethemocyanin Prepared by Means of  
Permanganate at pH 7

KMnO <sub>4</sub> employed*	Total copper†	Cupric copper		Source of oxyhemocyanin
		By K <sub>4</sub> Fe(CN) <sub>6</sub>	By C <sub>10</sub> H <sub>2</sub> O <sub>2</sub>	
equivalents	m -eq per cc	m -eq per cc	m -eq per cc	
1	1 10		0 42	Crude <i>Limulus poly-</i> <i>phemus</i> blood
1 8	1 10		0 66	
3 5	1 10		0 94	
5 3	1 10		1 01	
7 0	1 10		1 01	
5 0	0 77	0 86	0 76	Dialyzed and precipi- tated hemocyanin from <i>Limulus</i>
5 0	0 77	0 78	0 75	

\* Calculated on the assumption that a manganous salt is the product of reduction.

† Determined after decomposition with H<sub>2</sub>SO<sub>4</sub>-HNO<sub>3</sub> (the digestion must be complete) by the method of colorimetric comparison, with K<sub>4</sub>Fe(CN)<sub>6</sub>; (see Yoe (1)).

according to the method of Redfield, Coolidge, and Shotts (2). It was dissolved in a 0.1 M phosphate buffer of pH 7, so that the concentration corresponded to about  $1 \times 10^{-3}$  milli-equivalents of copper per cc. 50 cc. of such a solution were treated with either 10 cc. of 0.0077 M KMnO<sub>4</sub> or 20 cc. of 0.01 M potassium molybdicyanide. The solution was diluted with about 35 cc. of phosphate buffer and allowed to stand at ice temperature until the reaction was complete. Preliminary experiments had shown that this required less than 20 minutes in the case of potassium permanganate and 1 to 3 hours in the case of molybdicyanide; the course of the reaction could be followed by withdrawing samples (5 cc.) and titrating electrometrically with naphthohydroquinone (0.002 M). The naphthohydroquinone solution was prepared by the catalytic reduction of a naphthoquinone solution in phosphate buffer with palladium-asbestos and hydrogen; it was stored in an atmosphere of nitrogen. It was standardized against a carefully prepared solution of potassium ferricyanide. If potas-

sium permanganate is used in the preparation, a titration may also be carried out with potassium ferrocyanide (0.005 M) as the reducing agent. In this case 5 cc. are treated with 1 cc. of ferrocyanide solution and 3 cc. of buffer solution; the mixture is then titrated rapidly with 0.003 M  $K_2Mo(CN)_6$ , the end-point being determined electrometrically. The total copper was determined by digestion of a 3 cc. portion. 15 cc. were reduced with hydrosulfite and oxygenated with air; the blue color which resulted was compared in a colorimeter with a sample of the original oxyhemocyanin solution. If molybdicyanide is used, the blue color is attended by the presence of the yellow molybdocyanide and this procedure cannot be used. The reproducibility of the results is indicated by the typical analyses given at the end of this description.

After the reaction mixture had been analyzed as described above, it was centrifuged for 10 to 15 minutes if permanganate was employed. This removed a small amount of brownish precipitate. 2 volumes of saturated ammonium sulfate solution were now added (the temperature was kept at 0°), and the mixture centrifuged for 2 hours in a cold room. The precipitate of purified oxymethemocyanin was dissolved in 25 cc. of buffer solution. The analyses of some typical solutions thus prepared are given in Table II.

The course of a typical preparation is indicated by the following analytical figures, all expressed in terms of milli-equivalents  $\times 10^3$  per cc.

(a) *With  $KMnO_4$* —Crude reaction mixture (total volume 95 cc.)  $Cu^{++} = 0.25, 0.26$  (by  $C_{10}H_8O_2$ ), total Cu = 0.24; after centrifuging,  $Cu^{++} = 0.24, 0.23$ , total Cu = 0.25; after precipitating and redissolving in 25 cc. of buffer,  $Cu^{++} = 0.24, 0.26$ , total Cu = 0.23.

(b) *With Molybdicyanide*—Crude reaction mixture,  $Cu^{++} = 0.36, 0.39$ , total Cu = 0.44; after precipitating and redissolving in 25 cc.,  $Cu^{++} = 0.18, 0.14$ , total Cu = 0.14.

The quantitative data in regard to the purified samples are given in Table II. The last column records the colorimetric results obtained according to the procedure outlined above in which the oxyhemocyanin is regenerated. An inspection of Table II shows that within the limits of experimental error ( $\pm 10$  per cent) the total copper in the purified oxymethemocyanin is present as

cupric copper determined by either of the two methods employed. The last column affords evidence that on reduction and oxygensation the full color of oxyhemocyanin resulted from each of the samples. The facts presented in Table II, as well as those in Table I, demonstrate that in oxymethemocyanin we are dealing with a cupric compound.

Oxymethemocyanin is of a greenish blue color, the shade being distinctly different from that of oxyhemocyanin itself but of about the same degree of intensity. The presence of cupric copper in the compound can be shown by a number of qualitative tests as

TABLE II  
*Properties of Purified Oxymethemocyanin*

Source	Total copper	Cupric copper		Oxyhemocyanin, colorimetric after reduction and oxygensation
		By $K_4Fe(CN)_6$	By $C_{10}H_8O_2$	
	<i>m -eq per cc</i> $\times 10^3$	<i>m -eq per cc</i> $\times 10^3$	<i>m -eq per cc</i> $\times 10^3$	
By $KMnO_4$ from purified oxyhemocyanin	* 1 92	2 29	1 91	1 95
	* 1 99	2 32	2 00	
	0 226	0 236	0 260	0 222
	0 340		0 306	
	0 420		0 390	
By $K_2Mo(CN)_8$ from purified oxyhemocyanin	0 156		0 144	
	0 137		0 144	
	* 0 486		0 442	
	* 0 480		0 440	

\* Duplicate experiments.

well as by the quantitative titrations mentioned above. Oxymethemocyanin oxidizes reduced indophenol (colorless) to the colored indophenol. It oxidizes gum guaiac (made by Eimer and Amend) and benzidine sulfate with the formation of the characteristic colors which, however, are only transient because of further oxidation. When treated with potassium cyanide the color of oxymethemocyanin is destroyed and oxygen is evolved as in the case of the cuprous compound, oxyhemocyanin. Preliminary results indicate that a colorless cyanide complex is formed in the case of both the cuprous and cupric compounds.



*Methemocyanin*

The oxygen combined with the protein in methemocyanin may be removed by reducing the partial pressure of the oxygen above the solution of the compound. Repeated evacuations of a tonometer containing oxymethemocyanin followed by the introduction of oxygen-free nitrogen are necessary. After repeated evacuations and fillings of the apparatus the color of the solution fades almost completely. On readmission of oxygen, the color is restored.

Another method of preparing the colorless cupric compound, methemocyanin, is to start with deoxygenated hemocyanin (colorless) in a tonometer filled with nitrogen. 3 to 5 equivalents of dilute permanganate solution (all at pH 7) are then added. The permanganate color is rapidly destroyed and an almost colorless solution results. There is a small amount of brownish color, perhaps due to a trace of colloidal manganese compound. The colorless methemocyanin thus prepared or prepared by deoxygenation of oxymethemocyanin shows all the characteristic oxidation reactions of oxymethemocyanin. It oxidizes reduced indophenol and potassium ferrocyanide, naphthohydroquinone, and benzhidene sulfate. The quantitative determinations must, of course, be carried out in the absence of oxygen, as otherwise one is dealing with oxymethemocyanin and not methemocyanin.

The result of the titration in nitrogen of a sample of methemocyanin made by deoxygenation of a sample of purified oxymethemocyanin was as follows: milli-equivalents per cc. of oxidizing power by naphthohydroquinone (electrometric end-point)  $0.295 \times 10^{-3}$  and  $0.306 \times 10^{-3}$ , total copper  $0.340 \times 10^{-3}$  and  $0.323 \times 10^{-3}$ ; ratio of  $\text{Cu}^{++}$  to total Cu = 0.88, 0.95. It is evident that the colorless methemocyanin is a cupric compound.

Both oxymethemocyanin and methemocyanin itself slowly decompose on standing and are less stable than the corresponding cuprous compound. The solutions must therefore be kept at ice temperature and worked with within a day of their preparation.

Methemocyanin absorbs oxygen, forming the blue-green oxymethemocyanin previously described. The absorption may be qualitatively demonstrated by the admission of a solution to a given volume of air in a constant volume apparatus. To determine more accurately the amount of combined oxygen in oxymet-

hemocyanin we have used the method of Redfield, Coolidge, and Shotts (2) which was developed for oxyhemocyanin. Like the cuprous compound, oxymethemocyanin forms a colorless cyanide complex which has no affinity for oxygen; the addition of potassium cyanide to an oxymethemocyanin solution therefore evolves oxygen. A constant volume Van Slyke apparatus was employed. A sample of oxymethemocyanin prepared by permanganate in the usual way (but not purified by precipitation) gave the following figures: total Cu  $0.95 \times 10^{-3}$  mg. per cc.; cupric Cu  $0.91 \times 10^{-3}$ ;  $O_2$  evolved  $0.46 \times 10^{-3}$  (0.49, 0.43) mm per cc. Ratio of Cu to  $O_2 = 2.1, 2.0, 2.2$ .

It is evident from the figures just given that the ratio of combined oxygen to copper is the same in oxymethemocyanin as in oxyhemocyanin itself. For this reason, no gas is evolved when oxymethemocyanin is reduced with ferrocyanide in a Warburg apparatus and no gas is absorbed when oxyhemocyanin (in air) is oxidized with permanganate or molybdcyanide.

#### *Oxidation-Reduction Potential of the Hemocyanin System*

Potassium ferrocyanide is oxidized by the cupric compounds, methemocyanin and oxymethemocyanin. If the former is used, the experiment must be performed in nitrogen, of course. If 1 equivalent of oxymethemocyanin is added to 1 equivalent of ferrocyanide (in a phosphate buffer at pH 7), 10 minutes after adding the oxymethemocyanin, the potential rose to +0.542 from the initial potential of the ferrocyanide of +0.365 volt; after 1 hour the potential was +0.545. (The potential is expressed in terms of the normal hydrogen electrode, the European convention in regard to sign being employed.) The fact that the equilibrium may be approached from both sides is shown by the behavior of a mixture of ferricyanide and oxyhemocyanin. The initial potential of ferricyanide is +0.615 volt and is lowered to +0.565 volt 10 minutes after adding 1 equivalent of oxyhemocyanin; in 1 hour the potential was practically constant at +0.545 volt. In duplicate experiments, the final equilibrium potential was found to be  $+540 \pm 5$  millivolts. When methemocyanin or hemocyanin was used, and the electrochemical cell filled with nitrogen, very similar results were obtained. When the equilibrium was approached from the methemocyanin side, the final value was +563

$\pm 10$  millivolts and from the other side  $+525 \pm 15$  millivolts. If tungsti- and tungstocyanides are used in place of ferri- and ferrocyanides, equilibrium may also be approached from both sides with oxygenated or deoxygenated compounds. The equilibrium potential with 1 equivalent each of reagent and protein is  $+0.540 \pm 0.010$  volt both in nitrogen and in air. The fact that the oxygenated and deoxygenated compounds give essentially the same results is to be expected since both the cuprous and cupric compounds combine with oxygen.

The experiments just described provide convincing evidence of the reversibility of the hemocyanin-methemocyanin system and the oxyhemocyanin-oxy methemocyanin system. At first sight it is surprising that the equilibrium potentials are essentially the same whether the ferricyanide system ( $E_h^0 = 0.440$ ) or the tungsticyanide system ( $E_h^0 = 0.530$ ) is employed. The explanation is undoubtedly due to the large value of  $n$  in the usual electrochemical equation.

$$E_h = E_h^0 - \frac{RT}{nF} \ln \frac{[\text{reduced}]}{[\text{oxidized}]}$$

If the hemocyanin molecule had a molecular weight corresponding to the minimum value of 73,400 which corresponds to 2 copper atoms or 1 oxygen molecule, the value of  $n$  in the electrochemical equation should be 2. Using this value and the results given above, we calculate  $E_h^0$  from the ferricyanide experiment as  $+0.596$  and from the tungsticyanide experiment as  $0.558$ . The discrepancy between these numbers might be due to experimental error but is more probably an indication that the value of  $n$  in the electrochemical equation is much larger than the minimum value. For example, if the value of 6 is taken, the two results become  $0.561$  and  $0.537$  respectively, while if a value of 10 is taken, the two numbers are in fairly good agreement ( $0.554$  and  $0.544$ ). While these measurements cannot be taken as conclusive proof of the large value of  $n$ , they are very suggestive that a value at least as large as 10 should be taken. The difficult problem connected with the value of  $n$  in the electrochemical equation governing the oxidation of hemoglobin has been discussed in papers from this laboratory (3) and need not be considered again at this point. It is interesting, however, that if we take the molecular weight of

*Limulus* hemocyanin as determined by Svedberg (4) as 2,040,000 the value of  $n$  in the electrochemical equation should be approximately 56, provided there are no intermediates. Such a large value would put the two determinations of the oxidation-reduction potential from the tungsticyanide and ferricyanide experiments very close together (0.546 and 0.540 volt).

The direct titration of either hemocyanin or oxyhemocyanin with an oxidizing agent is not entirely satisfactory, although the results were sufficiently definite so that they provided us with the first definite clue that we were working with a cuprous-cupric reversible system. After each addition of oxidizing agent it is necessary to wait for a considerable time before the potentials become constant. If one waits until the change of potential with time is small and constant and records these points as equilibrium points, it is possible to obtain a titration curve. Such a titration curve shows a fairly definite "break" at a point corresponding to 1 equivalent of molybdicyanide per copper atom. The back titration with sodium hydrosulfite is more rapid but the potentials are considerably below those obtained with molybdicyanide. The number of equivalents, as before, corresponds to 1 reducing equivalent per copper atom within 10 per cent. The potentials determined by this titration method are much less satisfactory criteria of the reversibility of the system than the experiments mentioned previously. They indicate approximately the same value of the oxidation-reduction potential as obtained by the more accurate experiments with 1 equivalent of oxidizing or reducing agent. The difficulties with the titration method are undoubtedly due to the fact that each small increment of oxidizing agent reacts to some extent with the organic part of the protein molecule as well as with the copper. It is also probable that the cuprous-cupric protein compounds themselves do not impress a satisfactory potential upon the electrode. For all these reasons the direct titration procedure, which is so valuable in studying many oxidation-reduction systems, does not particularly lend itself to the case of hemocyanin.

In all these experiments we have confined our attention to the hemocyanin from *Limulus polyphemus*. A few experiments with the hemocyanin from the blood of the lobster, *Homarus americanus*, indicate that it also may exist in a cupric state and the

potential is not very different from that of the *Limulus* hemocyanin.

We have never succeeded in preparing methemocyanin or oxy-methemocyanin by the action of any catalyst on oxyhemocyanin. The potential of the oxygen electrode is sufficiently high so that oxyhemocyanin has the possibility of decomposing to give oxy-methemocyanin, but we have not as yet been able to bring this change about in the laboratory. Unless a catalyst could be found that would make the change rapid, there is little chance of success, since the slow autoreduction of the cupric compound would keep the protein in the cuprous condition.

In a preliminary paper from this laboratory (5), the isolation of a black material from the alkaline decomposition of *Limulus* hemocyanin was described. This substance contained sulfur and nitrogen and about 20 per cent of copper; we believed it to be a true prosthetic group. Further experiments, which will be published shortly, have shown that this material is a complex copper salt of a polypeptide and a sulfur-containing amino acid. It is almost certainly a true prosthetic group and probably bound to the protein by coordinate linkage of the copper (in a manner similar to the binding of heme in hemoglobin). The black prosthetic group is insoluble in 0.1 N aqueous alkali but in the presence of a third of the volume of pyridine dissolves, forming a greenish solution. Such a solution undoubtedly contains a complex soluble salt of the prosthetic group and pyridine; it is comparable to a parahematin solution (or in the reduced state to a hemochromogen). The titration of such a solution (0.0123 mm of Cu per cc.) with sodium hydrosulfite in nitrogen showed the presence of cupric copper and back titration with ferricyanide confirmed this (mm of Cu per cc. by  $K_3Fe(CN)_6$ , 0.0127). The oxidation-reduction potential from the mid-point of the not very satisfactory titration curves was +0.15 volt. This is much lower than the value for the protein compound but the comparison between these measurements was only possible in a strongly alkaline solution. It appears, however, that, as in the case of hemoglobin, a combination of the prosthetic group with the protein raises the oxidation-reduction potential.

In conclusion attention may be drawn to the fact that the oxidation-reduction potential of the hemocyanin system is extremely

high. With the exception of the complex cyanides of molybdenum and tungsten and potassium permanganate, methemocyanin and oxymethemocyanin are among the strongest reversible oxidizing agents known. Whether or not this has any biological significance further experiments alone can decide. It is clear that if the cupric compound functioned in any biological process it would not persist long in the cupric state but would be reduced by many of the substrates present in the biological fluids. We could therefore not expect to obtain direct evidence of the existence of the cupric compound in living tissue.

We wish to express our sincere appreciation of the invaluable cooperation of Professor A. C. Redfield, without which this work would not have been possible.

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## THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND PHYSIOLOGICAL RESPONSE

### IV. CONJUGATION OF SALICYLIC ACID WITH GLYCINE AND ITS ACTION ON URIC ACID EXCRETION

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The experimental studies on the fate of salicylic acid have yielded many conflicting results. In 1856, Bertagnini (1) reported that after ingesting salicylic acid he succeeded in isolating a compound consisting of glycine and salicylic acid which he named salicyluric acid because of its analogy to hippuric acid. Later investigators who also reported finding salicyluric acid are Nencki (2), Lesnik (3), Mosso (4), Bass (5), Baldoni (6), and Stockman (7). Hanzlik (8), however, failed to find salicyluric acid even though he carefully followed the various procedures of the previous investigators. He therefore seriously questioned the occurrence of salicyluric acid in the urine. Several years later Holmes (9) reported the isolation of the conjugated product from human urine. He furthermore criticized the Thoburn-Hanzlik method (10), which consists essentially in steam-distilling salicylic acid from urine strongly acidified with phosphoric acid, as unsatisfactory for determining salicyluric acid. From his quantitative studies Holmes concluded that salicylic acid is excreted in the ratio of 40 per cent free to 60 per cent combined with glycine. Holmes' work has in turn been adversely criticized by Johnson (11). Recently the writer (12) in studying the conjugation of substituted benzoic acids discovered the rule that the union of glycine with a carboxyl group attached to a benzene ring is markedly inhibited by substitution in the ortho position. Since salicylic acid is *o*-hydroxybenzoic acid, its conjugation with glycine should be inhibited. It was found, as was anticipated, that the excretion of salicyluric acid was exceedingly small. On continuing



the study of salicylic acid, a simple method for isolating salicyluric acid was developed, which is described in this paper. Further quantitative studies on the conjugation of salicylic acid and on its influence on the excretion of uric acid are also reported.

#### EXPERIMENTAL

Both the successful isolation of salicyluric acid and the quantitative determination of free and combined salicylic acid employed in this work are based on the fact that these compounds can be completely removed from urine with ether by means of a continuous extractor. The formol titration method previously described by the author (13) for hippuric acid yields equally satisfactory results for salicyluric acid, and the writer's adaptation of the Day and Taggard bromination method (14, 15) for total salicylic acid is simple and accurate. These methods have the advantage that they can be applied to small volumes of urine and that they have a high degree of accuracy yet are simple and rapid enough to be suitable for the analysis of routine hourly specimens.

In studying the conjugation of salicylic acid and its influence on the excretion of uric acid, the drug was always given orally in the form of the sodium salt. A light breakfast consisting of coffee and a cruller or toast was eaten 1 hour before the test. During the experimental period the subject was kept on a low protein and low purine diet. With a fairly well maintained standard diet, a remarkable constancy not only in the conjugation of salicylic acid but also on its stimulation of uric acid excretion was observed. Even after an interval of 1 year, the response to a fixed dose of the drug showed no greater variation than could be accounted for by the experimental errors inherent in the methods of analysis. Uric acid in the urine was determined by the Benedict-Hitchcock method (16).

*Isolation of Salicyluric Acid*—Since hippuric acid and related compounds, which are always present in the urine after a mixed diet, cannot be separated from salicyluric acid, it is necessary for the isolation of pure salicyluric acid to put the subject on a low protein and a fruit- and vegetable-free diet for 24 hours prior to the test. 2.3 gm. of sodium salicylate are given orally and the urine collected for 24 hours, or if more convenient, for a shorter period. During the experiment only milk, bread, and sugar are

allowed. If these dietary precautions are observed, pure salicyluric acid having the correct melting point is readily obtained, whereas on a mixed diet, the product although white and crystalline will not melt correctly even after repeated recrystallizations from water. The urine is made acid to litmus and concentrated on the water bath to about 100 cc. After the removal of the sediment, the urine is made acid to Congo red, and extracted with ether in a continuous extractor until the urine no longer gives a purple color with ferric chloride. After removing the ether by distillation, the gummy or crystalline residue is leached twice with hot toluene and once with chloroform. The crude product is dissolved in a small volume of boiling water, treated with decolorizing charcoal, filtered, and allowed to crystallize. Usually one recrystallization is sufficient to yield a colorless product but an additional simple extraction or washing with chloroform to remove traces of free salicylic acid may be necessary to obtain a pure compound. A yield of about 0.5 gm. was obtained. In one experiment, 0.61 gm. was isolated from a 24 hour specimen of urine which from analysis was known to contain 0.96 gm. of salicyluric acid.

The melting point was found to be  $167^{\circ}$ , corrected. The accuracy of the thermometer was tested by determining the melting point of pure salicylic acid. The present finding agrees with the melting point of the synthetic salicyluric acid as recorded by both Hanzlik and Holmes. Salicyluric acid crystallizes in the form of needle crystals which tend to be grouped in rosettes. Microscopically, the crystals differ from those of salicylic acid in being more lanceolated, while those of the latter compound have square ends. Salicyluric acid is practically insoluble in chloroform and toluene. It is very soluble in hot water and in cold water it is about four times as soluble as hippuric acid. The dry compound easily becomes electrically charged, a property also noted by Bondi (17) for the synthetic product. The compound gives a purple color with ferric chloride, and after hydrolysis gives a positive ninhydrin reaction. Salicyluric acid, like *p*-hydroxyhippuric acid, unites with 2 atoms of bromine, and like the para isomer, it gives on titration with standard sodium hydroxide a higher titration than the expected based on the monobasic acid. This indicates that the hydrogen of the hydroxy group of salicyluric acid is more acidic, *i.e.* ionized, than it is in salicylic acid. Bertag-

nini stated that he suspected that salicyluric acid is dibasic and Bondi also noted this property in the synthetically prepared compound. This is another illustration of how a change in one substituted group attached to the benzene ring can affect a second group.

*Analysis*—Titration with 0.1 N sodium hydroxide: sample, 0.1 gm.; found, 6.4 cc.; calculated (for monobasic acid), 5.15 cc.

Bromination: sample, 0.1 gm.; found, 0.159 gm. bromine; calculated, 0.164 gm.

Glycine: sample, 0.1 gm.; found, 0.0352 gm.; calculated, 0.0384 gm.

*Determination of Acetylsalicylic Acid*—A simple procedure for determining acetylsalicylic acid has been developed on the basis that this compound will not combine with bromine, whereas free salicylic acid, which is liberated on hydrolysis, will take up 3 atoms of bromine. Therefore, by determining the amount of bromine consumed before and after hydrolysis, one can calculate the quantity of acetylsalicylic acid. If salicyluric acid is present, a correction must be made since this compound takes up 2 atoms of bromine, but is also hydrolyzed. For the determination of acetylsalicylic acid in urine, 10 cc. samples are extracted, after the urine has been acidified, with ether in a continuous extractor. One sample is analyzed by the bromination method directly, care being taken to keep the solution cold and allowing only 15 minutes for the reaction. A second sample is hydrolyzed by refluxing with 5 per cent sodium hydroxide before applying the bromination method. From the difference in the two titrations, the amount of salicylic acid combined with acetic acid can be calculated. A small fraction of the acetylsalicylic acid will be hydrolyzed during the determination, but the amount is surprisingly small and can be adequately corrected by a blank obtained by making a determination with pure acetylsalicylic acid.

#### DISCUSSION

The human organism can synthesize salicyluric acid but the amount excreted is very small and is not markedly influenced by exogenous glycine, as can be seen in Table I. In agreement with Holmes, a definite ratio of free salicylic acid to salicyluric acid seems to exist, but this ratio varies with the dose of sodium

salicylate administered since the amount of uncombined salicylic acid increases more with increasing doses of the drug than the output of salicyluric acid. The excretion of salicylic acid is not rapid, contrary to the statement found in some text-books of pharmacology. Whereas the excretion of hippuric acid following

TABLE I  
*Conjugation of Salicylic Acid in Man After Varying Doses of Sodium Salicylate*

Subject Q ; weight, 52 kilos.

Time	Salicylic acid ingested								
	10 gm			20 gm			35 gm		
	Excreted			Excreted			Excreted		
	Free*	As salicyluric acid†	Total	Free	As salicyluric acid	Total	Free	As salicyluric acid	Total
hrs	gm	gm	gm	gm	gm	gm	gm	gm	gm
1	0 007	0 020	0 027	0 004	0 023	0 027	0 023	0 027	0 050
2	0 014	0 029	0 043	0 016	0 026	0 042	0 125	0 035	0 160
3	0 016	0 029	0 045	0 036	0 029	0 065	0 159	0 031	0 190
4	0 022	0 021	0 043	0 033	0 033	0 066	0 098	0 029	0 127
25	0 006	0 008	0 014	0 008	0 021	0 029	0 002	0 039	0 041
Total in 24 hrs	0 260	0 280	0 540	0 420	0 680	1 100	1 262	0 658	1 920

With 15 gm. gelatin									
1	0 025	0 009	0 034	0 021	0 011	0 032	0 033	0 027	0 060
2	0 033	0 021	0 054	0 026	0 049	0 075	0 091	0 032	0 123
3		0 026		0 033	0 032	0 065	0 134	0 042	0 176
4	0 031	0 022	0 053	0 027	0 038	0 065	0 148	0 039	0 187

\* Probably includes a small amount of salicylic acid conjugated with sulfuric acid and glucuronic acid.

† Corrected for a blank which was found experimentally to be equivalent to 0.020 gm. of salicylic acid per hour.

the ingestion of 2 gm. of benzoic acid is complete in 4 hours, the elimination of 2 gm. of salicylic acid is only 50 per cent complete in 24 hours.

It is probable that the importance of salicyluric acid has been overemphasized. It is certainly erroneous to conclude that the

pharmacological differences between benzoic acid and salicylic acid are due to the fact that the former is detoxicated readily with glycine while the latter is only to a very limited degree. Other ortho-substituted benzoic acids exhibit the same limited conjugation with glycine as does salicylic acid, yet they are therapeutically inactive. Thus, *o*-chlorobenzoic acid not only resembles salicylic acid rather closely in its physical properties such as solubility and ionization constant, but it is also excreted partly uncombined and is conjugated only to a very small extent with glycine. Nevertheless, it possesses none of the physiological actions of salicylic acid. In fact, while the latter stimulates the excretion of uric acid, the chloro compound actually depresses it.

Since other ortho-substituted benzoic acids suppress the excretion of uric acid, the specific activity of salicylic acid must reside in the hydroxy group. That the activity of salicylic acid is not primarily dependent upon the inability of the organism to conjugate it readily with glycine is well illustrated by the fact that exogenous glycine, which tends to augment the formation of salicyluric acid, actually increases the physiological action of salicylic acid as measured by uric acid excretion. Although glycine alone in relatively large amounts will increase the output of uric acid, it seems rather certain that the synergistic action is not the result of a simple summation. Thus, while 5 gm. of glycine have practically no effect on the excretion of uric acid, 1.5 gm. when given with 2 gm. of salicylic acid will produce a striking augmentation. An answer for this somewhat paradoxical influence of glycine can perhaps be found in the author's previous studies (18, 19). It was found that while aromatic acids such as benzoic acid and substituted benzoic acids diminished the hourly output of uric acid, excess glycine significantly prevented, at least partially, this depressing effect. It seems, therefore, probable that salicylic acid in common with the other substituted benzoic acids may possess a masked inhibitory effect on uric acid excretion besides its predominant stimulatory action. When, therefore, the inhibitory factor is counteracted by glycine, the resulting stimulating effect becomes definitely more pronounced as is shown in Table II.

While glycine intensifies the action of salicylic acid, it also tends to shorten the period of stimulation. Thus, it was found that



when 1 gm. of salicylic acid was given by mouth, the stimulation lasted approximately 4 hours, whereas, if glycine was supplied, the uric acid output returned to normal in 3 hours. It seems that glycine accelerates the excretion of salicylic acid sufficiently to bring about a quicker reduction of the concentration of the drug to the level at which it is no longer effective. No obvious explanation can be offered for the finding that salicylic acid stimulates the output of uric acid only after a certain concentration of the drug is present in the organism and that stimulation immediately ceases after the concentration falls below this crucial level. In repeated experiments the author has found that while 0.5 gm. of salicylic acid will produce no increase in the hourly output of uric acid, a 1.0 gm. dose will cause a very marked augmentation. A concentration of about 0.8 gm. seems to be required in the subject studied to bring about an increase in the excretion of uric acid. On giving 0.6 gm. intravenously, however, a stimulation was observed during the 1st hour, but although only 0.02 gm. of salicylic acid was excreted during that period, the excretion of uric acid dropped to normal during the 2nd hour. In this case the loss of stimulation seems to have been brought about by a redistribution of the drug in the body. It should be observed that the level of salicylic acid needed to affect uric acid excretion is distinctly higher than that required for mild analgesia, but much lower than the concentration necessary to bring about relief in acute rheumatic fever. Salicylic acid appears to act specifically on uric acid; it does not affect to any marked degree creatinine, urea, chlorides, or the other common constituents of urine. The excretion of uric acid is apparently independent of the volume of the urine, and frequently a diminished urine volume was observed when the elimination of uric acid was high. A concentration of 250 mg. of uric acid per 100 cc. of urine was found on several occasions.

Although much work remains to be done before the mechanism involved in the excretion of uric acid can be explained, the fact remains that the synergistic action between glycine and salicylic acid should find definite therapeutic applications. It will be interesting to find whether glycine will aid salicylic acid in stimulating the excretion of uric acid in cases of pathological retention. Perhaps glycine may even augment the therapeutic action of salicylic acid in rheumatic fever.

The results obtained on acetylsalicylic acid are interesting, for 1.5 gm., which is equivalent to 1.15 gm. of salicylic acid, produced practically the same stimulation on uric acid excretion as when 1 gm. of salicylic acid was fed, while 0.75 gm., equivalent to 0.57 gm. of salicylic acid, caused no stimulation. Since the author has previously shown that the replacement of the hydrogen of the hydroxy group in salicylic acid, as illustrated by *o*-methoxybenzoic acid, abolishes the physiological activity, one must assume that the stimulatory action of acetylsalicylic acid is due rather to the liberated salicylic acid than to the intact compound. Acetylsalicylic acid apparently undergoes rapid hydrolysis in the body; nevertheless, some of the compound enters the general circulation as proved by the fact that the compound can be detected in the urine during the 1st hour following its ingestion. This finding was also made by Hanzlik and Presko (20) who administered much larger doses than were employed in this study.

In attempting to explain the physiological action of salicylic acid on the basis of its chemical constitution, it should be noted that the compound possesses two active chemical radicals attached to a benzene ring. If it be permitted to borrow from immunology, one can look upon these as haptophore-like groups. It is probable that the carboxyl group, since it unites with glycine, can also unite with the amino group of a larger aggregate of amino acids, possibly with a protein. It has been noted by Vinci (21) that salicylic acid shows an elective action on the element of the blood with which it undergoes a relatively strong union. The hydroxy group of salicylic acid, on the other hand, can combine with glucuronic acid. Thus the possibility of a glucuronic acid-salicylic acid-protein compound must at least be considered in attempting to elucidate the physiological action of salicylic acid. Glucuronic acid combined with salicylic acid should act as a hapten very much like the glucoside and galactoside of *p*-aminophenol which Goebel and Avery (22) conjugated artificially with a protein and thereby produced specific antigens. It is well known that salicylic acid and, more commonly, acetylsalicylic acid can cause symptoms such as urticaria and edema which are typically allergic. Due probably to the small molecular weight of the theoretical salicylic acid hapten group, such actions are relatively infrequent. With cinchophen these reactions are more common, and from the recent work of Davis (23) it appears that an individual can become



sensitized to the drug and remain sensitive for over a year and probably longer. As the writer (19) has previously pointed out, experimental findings furnish strong indication that cinchophen in the body is oxidized to a hydroxy derivative; moreover, the position of the carboxyl group which is on the  $\alpha$ -carbon atom of the quinoline ring produces inhibition of the glycine conjugation similar to ortho substitution. Thus, cinchophen appears to have in common with salicylic acid, an acquired hydroxy group and a carboxyl group so situated that conjugation with glycine is inhibited. Since the molecule of cinchophen is larger and more complex, it should give rise to a more active hapten. This seems to be borne out clinically since urticaria and other allergic reactions from cinchophen are relatively common.

#### SUMMARY

1. Pure salicyluric acid was isolated from human urine.
2. A method for the determination of acetylsalicylic acid in urine is described.
3. The rate of salicylic acid excretion is dependent upon the concentration of the drug in the body. With increasing doses of salicylic acid, the excretion of free salicylic acid becomes definitely greater, while the output of salicyluric acid is only slightly affected. Exogenous glycine has little influence on the excretion of salicyluric acid.
4. The stimulatory effect of salicylic acid on uric acid excretion appears to be dependent upon a fixed concentration of the drug in the body. Below this crucial concentration, salicylic acid has no apparent influence on the output of uric acid.
5. The action of salicylic acid on uric acid elimination is strikingly augmented by glycine or foods rich in glycine. An explanation for this finding is offered, and the possible therapeutic applications of this synergism between glycine and salicylic acid are pointed out.
6. A theory based on the possibility that salicylic acid conjugated with glucuronic acid may act as a hapten is proposed as a possible explanation for some of the physiological properties of the salicylates.

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## NOTES ON A SHAFFER-SOMOGYI COPPER REAGENT

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Our interest in copper-iodometric reagents of general composition similar to those of Shaffer and Hartmann (1920-21) and Somogyi (1926) was aroused by the necessity of finding a solution more sensitive to sugars and less sensitive to the non-sugar material of human bloods and urines. The solution reported in this paper, while not ideal, has advantages over those we have previously used. From time to time in other papers (Harding, Selby, and Armstrong, 1932; Harding, Nicholson, Grant, Hern, and Downs, 1932; Harding and Grant, 1932-33), reference has been made to this reagent. It has also been used in the studies shortly to be published on the use of a series of yeasts in the analysis of sugar mixtures. The more general paper of Shaffer and Somogyi (1933), however,<sup>1</sup> renders superfluous any detailed report. Our experiments confirm from another angle many of the conclusions of these authors, and have revealed an unexpected inaccuracy in some of the possible applications.

Iodide-free Shaffer-Hartmann reagents, while more sensitive than iodide-containing reagents, become unstable, deposit cuprous oxide, and lose some of their sensitivity. Shaffer and Somogyi recommend the addition of a small amount of KI (1 gm. per liter) to improve the stability. Our solution of the difficulty has been to prepare the reagent in two parts: Solution A, containing the copper salt and Solution B, the remainder of the chemicals. The two solutions<sup>2</sup> keep for at least 3 months under cool conditions, and

<sup>1</sup> The authors are indebted to Professor P. A. Shaffer and the editors of the *Journal* for an advance knowledge of this work.

<sup>2</sup> Solution A consists of 13.0 gm. of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  per liter. Solution B contains  $\text{NaHCO}_3$  (anhydrous), 50 gm.;  $\text{Na}_2\text{CO}_3$  (anhydrous), 40 gm.; Rochelle salt, 24 gm.; K oxalate, 36.8 gm.; K iodate, 1.4 gm. Dissolve each

we are assured of the highest sensitivity possible to a reagent of that composition.

This separation has also enabled us to measure the pH of Solution B, after 5-fold dilution, by means of indicators. Whereas this is not a measurement of the actual pH of the complete copper reagent, it has the practical value of defining the solution in terms other than the percentage or molecular composition, and has enabled us to detect inaccuracies brought about by negligence in weighing, or by impurities. A further control also convenient is to measure the titration value to phenolphthalein and methyl orange.

Our general laboratory technique differs very little from that of Shaffer and Somogyi. We stopper our  $6 \times \frac{3}{4}$  inch tubes with non-absorbent cotton during the heating period and use 2.0 cc. of sugar solution and 2.0 cc. of copper reagent with the addition of proportionally lessened amounts of acid and iodide after the reduction. The titration figures are proportionally less than after the use of 5.0 cc. The smaller amount has been necessitated by the consecutive use of two, or sometimes more, separate yeasts on 10 to 15 cc. of sugar solution, with the determination of residual sugar after each yeast removal. The time of heating has been kept constant at 10 minutes. This gives nearly the maximal values for glucose and fructose. Shaffer and Somogyi have pointed out that other sugars react more slowly and the time of testing should be increased to obtain a complete series of maxima. Preliminary experiments, however, had convinced us that our conditions gave us a high sugar value, with a value for non-sugar constituents of blood and urine much lower than with other variations of copper-iodometric reagents we had tried.

Table I shows the reduction values of 2.0 cc. of sugar solution at varying concentrations. Provided the total reducing value be not too high, mixtures of sugars give an additive figure.

The reagent has been applied to Folin-Wu, Herbert-Bourne, and Zn (Somogyi) blood filtrates. We have used it to determine urinary

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salt separately in minimal amounts of water at 25°. Mix when cool. Add rinsings to make to 1 liter. Keep at 10-15°. Equal quantities of Solutions A and B form the reagent. It is almost identical with Somogyi's (1926) solution (Spannuth and Power, 1931). This reagent is Solution VI in Table III.

sugar after "clearing" with  $\text{Pb}(\text{OOC}\cdot\text{CH}_3)_2$  and  $\text{KH}_2\text{PO}_4$ ;  $\text{H}_2\text{SO}_4$  and Lloyd's reagent;  $\text{KH}_2\text{PO}_4$  and  $\text{MgO}$ ; and  $\text{HgSO}_4\text{-BaCO}_3$ . Fructose and mannose give higher values than standard when added to urine.

*Influence of Ammonium Salts*—Table II shows the estimation of varying amounts of glucose when  $(\text{NH}_4)_2\text{SO}_4$  is added to the solution. In the presence of 50 mg. of  $\text{NH}_3$  per 100 cc., if the glucose content is 1 mg. per cent or less, none can be detected. With smaller amounts of  $\text{NH}_3$ , the errors are smaller, but not incon-

TABLE I  
*Reduction Values of Sugar Solution at Varying Concentrations*

sugar	0.005 N $\text{I}_2$ for 20 cc sugar solution							
	d-Glu- cose	d-Fruc- tose	d-Man- nose	d-Galac- tose	d-Malto- se	d-Lac- tose	l-Arab- inose	l-Xylose
mg per 100 cc	cc	cc	cc	cc	cc	cc	cc	cc
30	5 13	5 26	2 96	4 01	2 02	2 05	4 02	3 77
20	3 44	3 42	1 67	2 49	1 35	1 32	2 70	2 49
10	1 72	1 68	0 85	1 24	0 65	0 66	1 33	1 24
5	0 86	0 81	0 42	0 62	0 30	0 33	0 64	0 60
2.5	0 43	0 40	0 21	0 31	0 15	0 16	0 32	0 30
1.25	0 21	0 20	0 09	0 16	0 08	0 08	0 15	0 15
0.625	0 10	0 10	0 04	0 08	0 04	0 04	0 08	0 07
1 mg at 10 mg per 100 cc	8 60	8 45	4 25	6 55	3 25	3 30	6 65	6 20

siderable. Similar errors have been noticed with our other Shaffer-Hartmann reagents, and with other sugars.

The importance of these findings cannot be doubted. Normal human urine contains 500 to 1000 mg. of  $\text{NH}_3$  per 1000 to 1500 cc. per day. Assuming as convenient a dilution of the urine of 1:10, previous to the microestimation of the sugar, this would bring the range of  $\text{NH}_3$  concentration from 3.3 to 10 mg. per 100 cc. With a 10 mg. per cent glucose solution, low reduction values begin at a concentration of 2.5 mg. per cent  $\text{NH}_3$ . Lloyd's reagent does not remove  $\text{NH}_3$  completely so it will be evident that the direct use of such reagents on urines, so treated, must give low results in many instances. Mercuric sulfate followed by  $\text{BaCO}_3$  removes the  $\text{NH}_3$ .

and so presumably gives correct results as the reagent is unaffected by moderate concentrations of urea. This possible source of error should not be overlooked when other copper reagents are being used.

It is unfortunately no proof of the accuracy of total sugar determination in the presence of ammonium salts that a reagent correctly estimates added sugar. Thus, in Table II, above the level of 2.5 mg. per 100 cc. of glucose, each *increment* of glucose is correctly estimated. In presence of 50 mg. of  $\text{NH}_3$  per 100 cc., the addition of 2.5, 5.0, and 10.0 mg. of glucose per 100 cc. causes an increased reduction represented by 0.43, 0.84, and 1.60 cc. of

TABLE II

*Showing Effect of Varying Amounts of Ammonium Salts on Varying Amounts of Glucose, and the Effect of Ammonia Removal by  $\text{KH}_2\text{PO}_4$  and  $\text{MgO}$*

The figures represent cc. of 0.005 N  $\text{I}_2$  for 2 cc. of the sugar solution.

Glucose	Water value	$(\text{NH}_4)_2\text{SO}_4$ = 50 mg $\text{NH}_3$ per 100 cc	After $\text{NH}_3$ removal	$(\text{NH}_4)_2\text{SO}_4$ = 25 mg $\text{NH}_3$ per 100 cc	After $\text{NH}_3$ removal	$(\text{NH}_4)_2\text{SO}_4$ = 12.5 mg $\text{NH}_3$ per 100 cc	After $\text{NH}_3$ removal
<i>mg. per 100 cc.</i>							
20	3 46	3 08	3 44	3 11	3 42	3 39	3 43
10	1 72	1 39	1 73	1 46	1 71	1 68	1 73
5	0 86	0 55	0 87	0 64	0 85	0 80	0 86
2.5	0 43	0 12	0 43	0 21	0 41	0 39	0 42
1.25	0 22	0 00	0 22	0 05	0 21	0 18	0 22
0.625	0 11	0 00	0 11	0 04	0 10	0 06	0 10
0.3125	0 05	0 00	0 06	0 02	0 06	0 00	0 04

0.005 N  $\text{I}_2$ . These figures would be regarded as satisfactory evidence of the ability of the reagent to estimate added glucose.

The removal of  $\text{NH}_3$  is effected as  $\text{Mg}(\text{NH}_4)\text{PO}_4$ . To 25 cc. of solution, containing not more than 50 mg. per cent of  $\text{NH}_3$ , add 0.5 gm. of  $\text{KH}_2\text{PO}_4$ . Then add 1.0 gm. of solid light calcined  $\text{MgO}$ , a little at a time, and shake for 15 minutes. Filter. The filtrate is free from  $\text{NH}_3$  as tested by Nessler's reagent. The filtrate is brought to pH 6.5 by 1 or 2 drops of 5 N  $\text{H}_2\text{SO}_4$  previous to the sugar estimation. The amounts of  $\text{KH}_2\text{PO}_4$ ,  $\text{MgO}$ , and the time are capable of variation to suit other conditions. Sugar recoveries after such treatment are quantitative (Table II).

*Influence of pH*—We have repeated Somogyi's (1926) experi-

ments on the influence of the carbonate-bicarbonate ratio on the reduction value of glucose, and extended it to a number of sugars and other substances of immediate interest. Of the three factors, time of heating, total concentration of carbonate, and carbonate-bicarbonate ratio, we have kept the first constant, and the second

TABLE III

*Showing Effect of Carbonate to Bicarbonate Ratio on Reduction Value (Cc. of 0.005 N  $I_2$  per 0.2 Mg of Sugar) of Various Sugars with Constant Time of Heating (10 Minutes)*

Solution No	I	II	III	IV	V	VI	VII	VIII	IX	X
Glucose	0 96	1 18	1 35	1 48	1 64	1 72	1 71	1 53	1 18	0 57
Mannose	0 87	0 82	0 80	0 83	0 89	0 85	0 82	0 71	0 60	0 41
Galactose	0 90	1 10	1 18	1 24	1 26	1 24	1 16	0 86	0 60	0 37
Maltose	0 47	0 54	0 58	0 64	0 65	0 65	0 53	0 42	0 30	0 19
Lactose ..	0 53	0 64	0 68	0 69	0 69	0 66	0 53	0 40	0 24	0 15
Arabinose	1 15	1 26	1 31	1 33	1 34	1 33	1 12	0 93	0 66	0 43
Xylose	0 78	0 98	1 06	1 18	1 23	1 24	1 20	1 05	0 74	0 45
Rhamnose	0 44	0 48	0 59	0 65	0 69	0 68	0 59	0 46	0 30	0 17
Fructose	0 90	1 10	1 24	1 40	1 53	1 68	1 73	1 80	1 84	1 77
Glucosamine HCl	1 00	1 21	1 37	1 52	1 63	1 73	1 80	1 83	1 79	1 54
Glucuronic acid	0 60	0 71	0 77	0 83	0 90	0 97	1 07	1 18	1 26	1 20
Cystine HCl	1 42	1 41	1 35	1 24	1 19	1 20	1 19	1 19	1 20	1 21
Glutathione	1 17	1 09	1 05	0 97	0 88	0 80	0 77	0 71	0 67	0 66
<hr/>										
Titration value of 5.00 cc Solution B, cc N HCl										
(a) Phenolphthalein	4 1	3 7	3 4	2 8	2 4	2 0	1 5	1 1	0 7	0 4
(b) Methyl orange	8 5	8 3	8 0	7 7	7 4	7 1	6 7	6 4	6 1	5 7
pH	11 8	11 1	10 6	10 3	10 1	9 8	9 5	9 2	8 9	8 1
$Na_2CO_3$ , gm per l	90	80	70	60	50	40	30	20	10	0
$NaHCO_3$	0	10	20	30	40	50	60	70	80	90
" M		6 3	2 8	1 5	1 0	0 6	0 4	0 2	0 1	

has varied only in a narrow range. With a constant pH and constant total carbonate concentration Shaffer and Somogyi have shown the almost constant final reduction value of all sugars provided the time of heating is sufficient. They have suggested the use of their conditions as an aid to sugar differentiation. Our conditions show up this possibility more sharply (Table III). Aldoses give a low reduction value with the higher pH. This rises to a maximum at a  $Na_2CO_3:NaHCO_3$  ratio, varying for each



sugar, but is passed when the ratio is 0.4 M. Mannose is an exception. It possesses two maxima, the first at high alkalinity, and the second in the range of the remaining aldoses. Fructose, glucosamine, and glucuronic acid show maximal reduction values at carbonate-bicarbonate ratios of 0.2:0.1 M. Their rate of oxidation is evidently very rapid at the lower pH. Contrary to these, cystine and glutathione show high reduction values with the higher alkalinity.

Our conclusions reinforce those of Shaffer and Somogyi that there is no one ideal copper solution for all sugars, though it may be possible to prepare one of good general utility. These authors have described two such good general reagents. The one described in this paper, we believe to be such. For specific research problems it would be better to prepare a reagent suited to the specific conditions. Thus reagents with a low alkalinity, coupled with a short period of heating, would exaggerate the presence of fructose, or glucosamine, or glucuronic acid. Reagents with a high alkalinity would favor mannose, cystine, and glutathione.

#### SUMMARY

A modification of Somogyi's (1926) copper solution is used as a good general sugar reagent.

The effect of alteration of the carbonate-bicarbonate ratio on the reducing value of a series of sugars, and some other reducing substances is shown.

Ammonium salts interfere with sugar determinations by the above reagents.

A method for removal of ammonium salts without alteration of sugar values is given.

Each "sugar" problem requires a reagent suited to the conditions.

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## THE SYNTHESIS OF ASPARTIC ACID

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No one of the methods proposed for the synthesis of aspartic acid is entirely satisfactory as a practical procedure. The synthesis, described in a recent paper (1) from this laboratory, has been utilized in preparing 50 to 60 gm. lots of recrystallized aspartic acid but even here the beginning reagents are expensive, the over-all yield is only about 30 per cent of the theoretical, and the process is time-consuming because of the number of steps involved.

In searching for a convenient and inexpensive method of synthesis it was apparent that reactions with malic, succinic, maleic, or fumaric acid were the most promising. It was expected that ammonia could be made to react satisfactorily with chlorosuccinic or bromosuccinic acid, prepared from any one of the four dibasic acids, or directly with maleic or fumaric acid to give aspartic acid in good yield. Various methods for the preparation of the intermediate bromosuccinic (2-6) and chlorosuccinic acids (7-9) have been reported, whereas Fischer and Raske (10) were able to prepare 2.2 gm. (16 per cent of theory) of crude aspartic acid from bromosuccinic acid and aqueous ammonia. In our experiments with malic and fumaric acids the yields of chlorosuccinic and bromosuccinic acids were poor and only the insoluble ammonium salts resulted when these acids were treated with liquid ammonia or with a saturated solution of the dry gas in alcohol, ether, or pyridine. The formation of ammonium bromosuccinate in ether is in accord with the observations of McMaster and Magill (11).

The direct addition of ammonia to maleic and fumaric acids has been investigated by Engel (12) and Stadnikoff (13). When the amination was conducted at 140-150° in aqueous or alcoholic solution, Engel obtained a 30 to 35 per cent yield of aspartic

acid, although the quantity of amino acid synthesized and the analytical evidence of its purity were not reported. Stadnikoff isolated 3 gm. of diethyl aspartate, 2 gm. of diethyl iminosuccinate, and a small amount of an unidentified third substance by the fractional distillation *in vacuo* of the esterified products resulting from the reaction of 20 gm. of fumaric acid and an excess of ammonia. Numerous experiments were performed in the present investigation in attempting to confirm Engel's work but the results were negative.

In view of the exhaustive investigations of Morsch (14), who has studied the reaction of ammonia with a number of unsaturated esters, we were encouraged to try the addition of ammonia to fumaric ester. Subsequently, it was found that this reaction had been investigated by Koerner and Menozzi (15) who obtained diethyl aspartate in addition to a small amount of a crystalline substance which they believe to be aspartimide,  $\text{COCH}_2\text{CHNH}_2\text{CO}$ .



In a later paper by Fischer and Koenigs (16) it was shown that this substance is more probably the isomeric diketopiperazine diacetamide (the diamide of the diketopiperazine of aspartic acid),



Koerner and Menozzi isolated aspartic acid from the alkaline hydrolysate of the crystalline product, but no yields were stated.

In our experiments it was found that alcoholic ammonia reacts with diethyl fumarate, in 24 hours at  $100^\circ$  and 6 atmospheres pressure, to give principally the crystalline diketopiperazine diacetamide. The latter is readily hydrolyzed by sodium hydroxide to aspartic acid which is conveniently isolated as the copper salt. A 78 gm. yield (59 per cent of theory) of analytically pure aspartic acid was prepared by the procedures described in the experimental part.

#### EXPERIMENTAL

*Diethyl Fumarate*—The method of Corson, Adams, and Scott (17) was used in preparing 419 gm. of diethyl fumarate from 348 gm. of Eastman's Practical fumaric acid.

*Diketopiperazine Diacetamide*—Glass, ginger ale bottles (Canada Dry) were used for the pressure reactions described below. Bottles of this type resisted a pressure of 7.3 atmospheres when tested at 100° with 25 per cent aqueous ammonia,<sup>1</sup> whereas the pressure of the experimental solution was only about 6 atmospheres at 100°. Bottles to be used for pressure reactions should be well wrapped with cheese-cloth to minimize the danger from flying glass in case of an explosion. An iron pipe, similar to that described by Dakin (19), was used in one pressure experiment but it proved to be unsatisfactory partly because of its weight and unwieldiness but mainly because the chemical reactions appeared to take an entirely different course than in glass, perhaps due to some catalytic effect of the iron.

82 gm. (4.8 mols) of dry ammonia gas and 172 gm. (1.0 mol) of diethyl fumarate, b.p. 213–215° uncorrected, were dissolved in 1100 cc. of 99.85 per cent ethyl alcohol. This solution was divided equally among five ginger ale bottles each fitted with a firmly wired rubber stopper and a yard of cheese-cloth wrapping. The bottles were immersed in a water bath which was heated to boiling and maintained at this temperature for 24 hours. The solid crust, which formed on the sides of the bottle, was separated from the liquid material and the latter was distilled to dryness *in vacuo*. The combined solids were practically free from oil indicating that the diethyl fumarate and the intermediate diethyl aspartate had been converted almost completely to diketopiperazine diacetamide. The latter crystallizes from water as needles which do not melt sharply but decompose when heated above 250°.

$C_8H_{12}N_4O_4$ . Calculated, N 24.6; found (Kjeldahl), 24.3, 24.5

*Copper Aspartate*—The crude diketopiperazine diacetamide was refluxed for 6 hours in an oil bath with 750 cc. of 6 N sodium hydroxide. The resulting solution was neutralized to methyl red with 6 N hydrochloric acid and filtered. After several unsuccessful attempts to crystallize aspartic acid directly from the filtrate the latter was treated with a hot solution of 220 gm. (10 per cent excess) of copper acetate monohydrate in 1450 cc. of distilled

<sup>1</sup> The pressure-temperature-concentration relations of aqueous ammonia are given by Mellor (18).

water. Crystals of the light blue copper aspartate began to form within an hour. As stated by Hofmeister (20) these crystals appear as tyrosine-like clusters when viewed under the microscope. After standing overnight in the ice box, the voluminous precipitate was filtered and a sample recrystallized from water. When dried to constant weight *in vacuo* over sulfuric acid, the purified salt appeared to be the trihydrate, a finding in agreement with the observations of Cook and Woolf (21).

$C_4H_8O_4NCu \cdot 3H_2O$ . Calculated, N 5.63; found (Van Slyke), 5.60, 5.60

*Aspartic Acid*—The crude copper aspartate was suspended in 2 liters of 1 N acetic acid in a 4 liter flask and saturated with hydrogen sulfide. By maintaining a slight pressure of the hydrogen sulfide and intermittently shaking the flask, the decomposition of the copper salt was completed in about 2 hours. The resulting suspension was boiled gently for about 30 minutes, the coagulated cupric sulfide filtered, and the filtrate evaporated until crystals began to separate. After adding 3 liters of 95 per cent ethyl alcohol and standing overnight in the ice box the resulting crystals of aspartic acid were filtered and dried for 24 hours at 55°.

The mother liquor was distilled under reduced pressure until solid began to separate. Then about 1 liter of distilled water was added, the solution distilled to dryness *in vacuo*, and the residue converted to aspartic acid through the copper salt as previously described.

The yield of analytically pure aspartic acid was 64 gm., first crop, 14 gm., second crop, or a total of 78 gm. (59 per cent of theory). The weight of crude amino acid was 129 gm. (96 per cent of theory) and of once recrystallized material 101 gm. (76 per cent of theory). It is probable that the yield of analytically pure material can be increased since some decomposition occurred during the vacuum distillations.

$C_4H_7O_4N$ . Calculated, N 10.53; found (Van Slyke), first crop, 10.46, 10.46, 10.68, 10.73; second crop, 10.54, 10.62, 10.69

#### SUMMARY

It has been shown that analytically pure aspartic acid may be conveniently and inexpensively synthesized from diethyl fumarate

and alcoholic ammonia through the intermediate diketopiperazine diacetamide and copper aspartate.

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## THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

### XXXIII. ISOLATION OF TREHALOSE FROM THE ACETONE-SOLUBLE FAT OF THE HUMAN TUBERCLE BACILLUS\*

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(Received for publication, May 24, 1933)

#### INTRODUCTION

It has been shown in previous investigations in this laboratory that the acetone-soluble fats from the human (1) and bovine (2) tubercle bacilli and the timothy bacillus (3) are mixtures of free fatty acids and neutral fat. The neutral fats from acid-fast bacteria, in common with other fats, have been regarded as glycerides but in no case has it been proved definitely that such fats are true glycerides (4). In our earlier analyses, we were unable to identify glycerol although some water-soluble compound, different from glycerol, was obtained after the fats had been saponified. We believed, therefore, that the neutral bacillary fats were not glycerides but esters of fatty acids with some higher polyhydric alcohol, or possibly with some carbohydrate.

The human type of tubercle bacillus, Strain H-37, used in this work had been grown on the Long synthetic medium (5) at the Mulford Biological Laboratories, Sharp and Dohme, and the acetone-soluble fat had been isolated by the procedure described in the first paper of this series (6).

Our primary object in this investigation was to secure a larger quantity of the liquid saturated fatty acids, tuberculostearic acid

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† Holder of a National Tuberculosis Association Fellowship at Yale University, 1932-33.



and phthioic acid, but since we had a relatively large amount of the acetone-soluble fat at our disposal, an effort was also made to identify the polyhydric alcohol contained in this fat. The substance in question was found to be the crystalline disaccharide, trehalose.

It has been shown previously in this laboratory that the free polysaccharide fraction, contained in the alcohol-ether extract of the timothy-grass bacillus (7), contains a relatively large amount of trehalose. The presence of free trehalose in microorganisms is not surprising because bacteria belong to the group of chlorophyll-free plants known as fungi and trehalose is the characteristic sugar occurring in this type of plant. So far as we are aware, however, this is the first time that trehalose has been found in place of glycerol as the alcoholic component of a fat.

#### EXPERIMENTAL

*Isolation of the Water-Soluble Constituents*—The acetone-soluble fat, about 260 gm., was saponified by refluxing for 4 hours with an excess of alcoholic potassium hydroxide. The unsaponifiable matter and the fatty acids were isolated in the usual manner and reserved for future investigations.

The aqueous solution, from which the fatty acids had been extracted after acidification with hydrochloric acid, was neutralized with potassium hydroxide and concentrated under reduced pressure until a large amount of potassium chloride separated. The potassium chloride was filtered off, washed with alcohol, and discarded. The filtrate was concentrated under reduced pressure to dryness. The residue was extracted several times with warm absolute alcohol and the insoluble portion consisting largely of potassium chloride was collected on a Buchner funnel and washed with alcohol. It was noticed that there adhered to the potassium chloride crystals some sticky syrupy material which was insoluble in absolute alcohol and this substance was removed, as will be described later.

The alcoholic solution on evaporation to dryness left a syrupy residue which, after it had been dried in a vacuum desiccator, weighed 3.5 gm. The syrup was easily soluble in water and in alcohol but closer examination showed that it represented incompletely saponified material. After the substance had been refluxed

a second time for several hours with alcoholic potassium hydroxide, a small amount of unsaponifiable matter and about 3 per cent of fatty acids were obtained.

After this treatment the water-soluble substance was again isolated and, on drying in a vacuum desiccator, it formed a solid resinous mass. The material was thoroughly triturated with acetone in order to remove any glycerol that might be present. The acetone extract was evaporated to dryness, when a very slight residue was obtained. The residue when heated with acid potassium sulfate did not give the characteristic odor of acrolein and we may conclude, therefore, that glycerol was absent. The acetone-insoluble portion could not be crystallized and we were unable to prepare any crystalline derivative from it; hence, we do not know anything concerning the composition of this fraction. The aqueous solution of the substance gave no reduction with Fehling's solution either before or after boiling with dilute acid. The material may represent a partial decomposition product of the crystalline carbohydrate which was isolated, as will be described later.

It was mentioned above that a syrupy substance insoluble in absolute alcohol adhered to the potassium chloride crystals. The syrupy material was dissolved when the crystalline mass was treated with ice-cold 50 per cent alcohol. The insoluble potassium chloride was filtered off and washed with alcohol. The filtrate was evaporated to dryness *in vacuo* and the residue was treated with cold 60 per cent alcohol. A small amount of insoluble potassium chloride was filtered off and the filtrate was again evaporated to dryness *in vacuo*. The residue was dissolved in water and the solution was precipitated by adding a slight excess of neutral lead acetate. The insoluble lead precipitate, which consisted largely of lead chloride, was filtered off, washed with water, and discarded.

To the filtrate were added an excess of basic lead acetate and ammonium hydroxide, when a heavy white amorphous precipitate separated. After the mixture had stood overnight, the precipitate was filtered off, washed with dilute ammonia, and dried in a vacuum desiccator over sulfuric acid.

The lead compound was suspended in water, decomposed with hydrogen sulfide, filtered, and the nearly colorless filtrate was concentrated *in vacuo* to a thick syrup. The syrup was treated with

absolute alcohol and yielded 3.74 gm. of a white amorphous powder. The alcoholic solution on concentration to dryness left 1.5 gm. of a syrupy residue. The total amount of water-soluble constituents that were recovered was, therefore, 8.74 gm. which is equal to 3.3 per cent of the fat.

*Examination of the Alcohol-Insoluble Fraction*—The white amorphous powder referred to above was evidently a polysaccharide. The aqueous solution of the substance gave no reduction when boiled with Fehling's solution but after it had been heated for some time with dilute acid it gave a strong reduction. The substance gave no pentose or ketose color reaction.

A preliminary examination was made of the reducing sugars which were liberated after 1.5 gm. of the substance had been refluxed for 2.5 hours with dilute sulfuric acid. No mannose could be found but an osazone was easily formed in a yield of 88 per cent. After the osazone had been recrystallized from dilute alcohol, it melted with decomposition at 208° and there was no depression of the melting point when some of the substance was mixed with pure glucosazone.

A portion of the hydrolysate, freed of sulfuric acid, when evaporated to dryness, left a syrupy residue which on standing and scratching crystallized. The mixture was stirred up with 95 per cent alcohol, after which the crystals were filtered off, washed with alcohol, and dried *in vacuo*. Some of the crude crystals when dissolved in water showed a specific optical rotation of +49.0°, which value approaches that of glucose. It would appear from the results obtained that only one reducing sugar, namely glucose, had been formed when the polysaccharide was hydrolyzed.

*Purification of the Polysaccharide. Isolation of Trehalose*—The balance of the amorphous powder was dissolved in a little warm water; the solution was treated with norit, filtered, and concentrated *in vacuo*. Colorless crystals separated slowly from the syrup on standing and scratching. The mixture was stirred up with alcohol and the crystals were filtered off, washed with alcohol and ether, and dried in the air. The product which weighed 1.2 gm. was dissolved in 2 cc. of warm water and the solution was diluted with 10 cc. of absolute alcohol. A slight cloudiness which appeared on adding the alcohol was removed by filtration. Crys-

tallization was started by scratching and, on standing in the ice box overnight, large, colorless, transparent, rhombic crystals separated. The crystal form was identical with that of recrystallized commercial trehalose. The product was recrystallized in the same manner and after drying in the air it melted at 97–98°. There was no depression of the melting point when some of the substance was mixed with recrystallized commercial trehalose.

On drying to constant weight at 105° *in vacuo* over dehydrite, the loss in weight was 9.08 per cent, corresponding to 2 molecules of water of crystallization.

*Rotation*—0.1230 gm. of the air-dried crystals was dissolved in water and made up to 10 cc. In a 1 dm. tube  $\alpha = +2.263^\circ$ ;  $[\alpha]_D^{21} = +183.9^\circ$ . The calculated rotation of the water-free sugar is, therefore,  $+202.2^\circ$ .

*Preparation of the Octaacetate*—About 0.3 gm. of the crystallized trehalose was refluxed with 15 cc. of acetic anhydride and 0.5 gm. of fused sodium acetate for 2 hours. After the reaction mixture had cooled, it was poured into water and shaken until the acetic anhydride was decomposed. The acetyl derivative was extracted from the solution with chloroform and, after the chloroform extract had been washed free from acid with water, it was evaporated to dryness *in vacuo*. The acetyl derivative on recrystallization from methyl alcohol was obtained in the form of colorless prismatic needles which weighed 0.2 gm. and melted not sharply at 80°. The melting point and properties of the substance were identical with those of the octaacetate of commercial trehalose prepared under similar conditions.

*Rotation*—0.1228 gm. of substance was dissolved in chloroform and made up to 10 cc. In a 1 dm. tube  $\alpha = +2.006^\circ$ ;  $[\alpha]_D^{22} = +163.3^\circ$ .

#### SUMMARY

An investigation has been made of the water-soluble constituents which are liberated when the acetone-soluble fat from the human tubercle bacillus is saponified and it has been found that the disaccharide trehalose is present.

Trehalose itself was isolated in crystalline form and its octaacetate was prepared.

The neutral fat from the human tubercle bacillus which is

soluble in cold acetone is, therefore, not a glyceride but a complex ester of fatty acids with trehalose.

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## AMINO ACID NITROGEN IN BLOOD AND ITS DETERMINATION

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In 1922 Folin (1) proposed a method for the colorimetric determination of the  $\alpha$ -amino nitrogen of the amino acids. This method is based on the production of a yellow-orange color by the reaction of the amino acids with  $\beta$ -naphthoquinonesulfonic acid in alkaline solution. The intensity of the color produced is compared with the color produced by a known amount of glycine treated in the same manner. This method was developed mainly for the determination of  $\alpha$ -amino acid nitrogen in blood filtrates and urine but is applicable to other fluids containing amino acids.

The method has been widely used in the study of amino acids occurring in the blood stream. In general, satisfactory results have been obtained. During the course of a study on the concentration of  $\alpha$ -amino acid nitrogen in whole blood based on the analysis of unaltered blood filtrates, it was noticed that the range of true proportionality over which the color comparisons could be made was relatively small. Upon investigation it was found that this limitation was entirely due to the presence of a strong blank caused by the incomplete bleaching of the excess quinone reagent.

Edgar (2) found that by adding varying amounts of alkali to a series of determinations on the same sample of filtrate a variety of results was obtained. Re and Potick (3) reported failure in obtaining satisfactory results by this method. The results of Edgar are easily explained by the variable blank present which thus gave an apparent increase in the amino nitrogen content of the samples analyzed. Undoubtedly the presence of this strong blank was a factor in the unsatisfactory results reported by Re and Potick. However, their results were at such variance with all other published results that some other serious error must have been included in their work also.

It is the purpose of this paper (1) to describe a modification of the original Folin method; this modification will give determinations containing no blank, thereby increasing the range of true proportionality; (2) to present evidence showing that the tungstic acid filtrates prepared from unlaked blood give true values for the free amino acid nitrogen in the blood stream and that the values found in laked blood filtrates are much too high; and (3) to record amino acid nitrogen values found in the blood of normal men and animals when this analysis is based on the unlaked blood filtrate.

*Description of Modification Proposed in Amino Acid Nitrogen Method*

The principle of the modified procedure is the same as that of the original method, but each solution except the  $\beta$ -naphthoquinone sulfonic acid has been changed in detail. These changes will be described and the reasons for each will be briefly stated.

The solutions used were the following.

*Standard Amino Acid Solution*—Two stock solutions are prepared; one contains 0.1 mg. of amino nitrogen per cc. as glycine dissolved in 0.07 N HCl plus 0.2 per cent sodium benzoate, and the other contains 0.1 mg. of amino nitrogen per cc. as glutamic acid in 0.07 N HCl plus 0.2 per cent sodium benzoate. The sodium benzoate is used as a preservative. Standards used in the analysis of blood filtrates are prepared from these by mixing equal volumes of the two stock solutions and diluting with 0.07 N HCl containing 0.2 per cent sodium benzoate to a concentration of 0.03 mg. and 0.05 mg. of amino nitrogen per cc. A 0.03 mg. standard is prepared by adding 15 cc. each of the glycine stock solution and the glutamic acid stock solution to a 100 cc. volumetric flask. The contents are made up to a volume of 100 cc. with 0.07 N HCl containing 0.2 per cent sodium benzoate. The 0.03 mg. standard is used for the analysis of filtrates prepared from normal human unlaked blood. The amino acid concentration in most animal bloods is appreciably greater than that of human blood, therefore the strength of the standard used for such comparisons should be increased accordingly. It is not permissible to use 2 cc. of a weaker standard to prepare a standard of double strength due to the fact that the standard is so prepared that 1 cc. contains about the equivalent of acid found in 10 cc. of tungstic acid blood filtrate.

If a stronger standard is required it is necessary to prepare it from the stock solutions.

The use of both glycine and glutamic acid in the standard is made necessary because the shades of color produced by the different amino acids vary somewhat. The standard as recommended produces a color that matches very nearly that produced in the tungstic acid blood filtrates.

*Borax Solution*—As a source of alkali a 1.5 per cent solution of borax is used. Borax is used instead of the sodium carbonate solution for with its use less blank remains and the results are more satisfactory under the conditions recommended. A definite volume (2 cc.) of borax is added to each determination.

*Bleaching Reagents*—Two solutions are needed for the bleaching of the excess  $\beta$ -naphthoquinonesulfonic acid reagent: (1) a 0.1 M solution of sodium thiosulfate which need not be standardized, and (2) an acid formaldehyde solution prepared by mixing 3 volumes of 1.5 N HCl and 1 volume of glacial acetic acid with 4 volumes of 0.15 M formaldehyde. The 0.15 M formaldehyde solution may be prepared accurately enough by diluting 11.3 cc. of ordinary 40 per cent formaldehyde to 1000 cc.

Sodium thiosulfate in acid solution has the property of destroying the color of  $\beta$ -naphthoquinonesulfonic acid. If acetic acid is used in making this solution acid the bleaching of the quinone is far from complete but if a strong acid like hydrochloric acid is used the color is bleached completely. However, in the presence of a strong acid, sodium thiosulfate immediately decomposes with the liberation of sulfur. The addition of an amount of formaldehyde equivalent to the thiosulfate present will delay this decomposition for many hours. In the solutions as recommended the thiosulfate is present in a slight excess over the formaldehyde, *i.e.* 2 cc. of 0.1 M thiosulfate and 2 cc. of 0.075 M formaldehyde. Under these conditions the bleaching is more prompt and complete, and the solution remains perfectly clear for several hours.

The addition of acetic acid to the acid formaldehyde is made necessary because an amino acid determination made on a solution containing tryptophane will become cloudy in the absence of acetic acid. Tryptophane is the only amino acid we have studied that behaves in this manner. Determinations on unclaked blood filtrates develop a very slight cloud if acetic acid is not included in



the acid formaldehyde solution. This cloud is presumably due to tryptophane present in the filtrate.

*Sulfate-Tungstate Solution for Addition to Standard*—This solution contains 15.0 gm. of  $\text{Na}_2\text{SO}_4$  (anhydrous) and 1.5 gm. of  $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$  per 1125 cc. of solution.

The unlaked blood filtrate contains about 1.5 per cent  $\text{Na}_2\text{SO}_4$  and about 0.15 per cent  $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$  which remains from the solutions used in the preparation of the filtrate. The presence of these salts in the above concentrations has very little or no effect on the amount of color developed in the determination but sodium tungstate in particular and sodium sulfate to a much smaller degree alter the shade of color produced, making exact color comparisons difficult. The presence of sodium tungstate produces a disturbing greenish shade. 9 cc. of the above solution are, therefore, added to the standard to balance this effect and to bring the volume of the standard up to that of the unknown.

If sodium sulfate is present in concentrations of 3 per cent or more, or sodium tungstate in concentrations of 0.6 per cent or more, an error of 2 per cent or more, depending on the concentrations, will be introduced.

*$\beta$ -Naphthoquinonesulfonic Acid Solution*—We are still using this solution as originally recommended, that is, a freshly dissolved 0.5 per cent solution of  $\beta$ -naphthoquinonesulfonic acid in water. The solid reagent is prepared according to the directions given by Folin.

#### *Description of Determination*

The amino acid nitrogen content of a blood filtrate may be determined on either 5 cc. or 10 cc. of filtrate. When 10 cc. of filtrate are used the procedure is as follows:

Transfer 10 cc. of the filtrate into a test-tube graduated at the 25 cc. mark. To this add 2 cc. of 1.5 per cent borax solution and 2 cc. of a freshly prepared 0.5 per cent  $\beta$ -naphthoquinonesulfonic acid solution and mix thoroughly. The standard is prepared by introducing 1 cc. of the standard solution of desired strength into a test-tube similar to that used for the filtrate. To the standard that is to be used in the comparison of an unlaked blood filtrate or plasma filtrate add 9 cc. of the sulfate-tungstate solution prepared for this purpose. If the filtrate to be analyzed contains no tungstic

acid and very little sodium sulfate (laked blood filtrate), add 9 cc. of water. Then add to the standard 2 cc. of the 1.5 per cent borax solution and 2 cc. of the 0.5 per cent  $\beta$ -naphthoquinonesulfonic acid solution and mix well. Both standard and unknown are then set in a dark closet for 18 to 24 hours. After this period of standing add 2 cc. of the acid formaldehyde solution and 2 cc. of the 0.1 M sodium thiosulfate solution. Dilute the contents of each tube to a volume of 25 cc. with distilled water and then mix thoroughly. After standing for 4 to 5 minutes to allow for the complete bleaching of the excess quinone reagent, the unknown is compared with the standard by the use of a colorimeter.

If 5 cc. of the blood filtrate are used for the determination, add 1 cc. of the borax solution and 1 cc. of the 0.5 per cent quinone solution to the filtrate. The standard is prepared as described above. After the 18 to 24 hour period of standing add 1 cc. of each the acid formaldehyde and the thiosulfate solutions to the unknown, dilute to 15 cc., and mix. To the standard add 2 cc. portions of the acid formaldehyde and thiosulfate solutions, dilute to 30 cc., and mix. Color comparisons are made as directed above.

The calculation in either case is made by the use of the following formula,

$$20/R \times 0.03 \times 100 = \text{mg. per cent or } 20/R \times 3 = \text{mg. per cent}$$

when the colorimeter setting for the standard is 20.  $R$  is the reading of the unknown and 0.03 mg. is the concentration of the amino nitrogen in the standard.

When the amino acid nitrogen of fluids other than blood filtrates is to be determined, the sample taken should contain between 0.03 and 0.15 mg. of amino nitrogen and should be neutralized with sodium hydroxide or hydrochloric acid depending on whether the sample is acidic or basic, phenolphthalein being used as indicator. In order to balance the acidity of the unknown with the standard add 1 cc. of 0.07 N HCl. The determination is then carried out in the same manner as described for blood filtrates.

*Amount of Color Developed by Various Amino Acids*—The amount of color developed by the various samples of pure amino acids which we had on hand was compared with that produced by two of the amino acids (*i.e.* glycine and glutamic acid) taken as standards. In Table I are tabulated the results found. The

shade of color developed by some of the amino acids is somewhat different from that produced by others. In the last column of Table I an attempt is made to indicate how these shades appear in the colorimeter when compared with the standard. In comparing the color produced by two amino acids, which show a difference in shade, the criterion for the comparison should be the matching of

TABLE I

*Values Obtained from Different Amino Acids Compared with Glycine and with Glutamic Acid As Standards*

Standard	Experiment No	Amino acid	NH <sub>2</sub> -N		Average error	Shade compared with standard
			Added	Found		
			mg	mg	per cent	
Glycine	1	Alanine	0 075	0 0754	0 5	Shades good
	2	Cystine	0 075	0 073	2 7	Greenish yellow
	3	Lysine	0 0375	0 0355	5 3	Much yellower
	4	Tyrosine	0 075	0 0741	1 2	Yellower
	5	Histidine	0 075	0 0746	0 5	"
	6	Aspartic acid	0 075	0 0743	0 93	"
	7	Glutamic "	0 075	0 0748	0 26	"
	8	Leucine	0 075	0 0748	0 26	Shades good
	9	Tryptophane	0 075	0 075	0 0	Little grayish
	10	Serine	0 07	0 07	0 0	Shades good
Glutamic acid	1	Alanine	0 075	0 0765	2 0	Purplish tan tinge
	2	Cystine	0 075	0 0721	3 8	Gray tinge
	3	Lysine	0 0375	0 0365	2 7	Much yellower
	4	Tyrosine	0 075	0 0748	0 26	Little "
	5	Histidine	0 075	0 0761	1 4	Shades good
	6	Aspartic acid	0 075	0 0745	0 6	" "
	7	Glycine	0 07	0 0706	0 86	Purplish tan tinge
	8	Leucine	0 075	0 076	1 3	Shades good

the amount of yellow color in each and not the intensity of light allowed to pass through the solutions.

While this difference in the shade of color developed by two different amino acids may be quite disturbing when viewed in a colorimeter, it should not be stressed unduly for it is not great. No difference in shade can be noted when two determinations in test-tubes are compared with the naked eye. However, it is

well in analyzing for amino acid nitrogen to choose a standard which matches exactly with the unknown. This will make color comparisons easier and the error introduced in judging the exact comparison by different individuals will be reduced. It is because the shade of color developed by a blood filtrate is somewhat different from glycine or any other amino acid easily obtained in the pure state, that we are recommending the use of a standard containing one-half of its nitrogen as glycine and one-half as glutamic acid. This standard matches exactly a blood filtrate when used as recommended.

A solution containing equal amounts of amino nitrogen of the amino acids tested in Table I produces a shade of color that matches very well with that produced by the standard recommended for blood analysis and the proportionality is perfect.

In this connection it should be mentioned that ammonia reacts with  $\beta$ -naphthoquinonesulfonic acid under the conditions recommended and must therefore be removed from a solution to be analyzed for amino acid nitrogen. The color produced by ammonia has a decidedly purplish tint when compared with the color produced by any of the amino acids. It is impossible to get a good color comparison between ammonia and an amino acid but ammonia seems to develop from 65 to 75 per cent of the color developed by an equivalent amount of glycine.

#### *Amino Acid Nitrogen in Whole Blood*

In 1930, Folin (4) proposed the use of a filtrate prepared from whole blood without hemolyzing the red corpuscles, as the basis for the study of food products and waste products occurring in the blood stream. The preparation of such a filtrate was accomplished by simply diluting the blood with a slightly hypertonic solution (1.5 per cent) of anhydrous sodium sulfate instead of distilled water. The amino acid nitrogen found in a filtrate prepared in this manner is much less than that found in a filtrate prepared by laking the red corpuscles; *i.e.*, the Folin-Wu tungstic acid filtrate.

In 1931, Simon (5) reported a study of the amino acid nitrogen content of whole blood based on the analysis of both laked and unlaked blood filtrates. He came to the conclusion that the value found in the unlaked blood filtrate was too low and that for this

determination, at least, the unlaked blood filtrate cannot be used to give accurate results.

Simon presents two very plausible arguments along with experimental data which apparently seem to uphold his conclusions. First, he makes the very reasonable suggestion that if in the preparation of an unlaked blood filtrate the free amino acids are freely diffusible to the extent characteristic of the blood, then on the addition of amino acids to the whole blood it should be expected that the increase in the amino nitrogen content of the red corpuscles, above that present before the addition of the extra amino acid, should be the same whether this value is calculated from data obtained from the analysis of filtrates prepared from laked blood or from unlaked blood. His experimental data show that this, apparently, is not the case. In the seven experiments reported, he always found a greater increase in the corpuscle value calculated on the laked blood analysis. He found that the increase in the corpuscle values calculated on the unlaked blood filtrate data was 40.2, 17.7, 74.3, 94.0, 32.7, 66.0, and 18.5 per cent respectively of the increase in the value based on laked blood data. In only one case did he find increases that were comparable; *i.e.*, the increase found in the unlaked corpuscles was 94 per cent of that found in the laked corpuscles.

Although Simon does not state exactly how much amino acid was added in each experiment, it can be seen from his tables that complete recovery of the added amino acids was not obtained in the majority of these experiments for in only one case is the increase in the whole blood nearly the same in both the laked and unlaked blood filtrates. This one case is the experiment in which nearly equivalent increases in the amino nitrogen content of the corpuscles were found.

We have repeated these experiments, using our modified amino acid nitrogen method, and in every case where the recovery of the added amino acids to the whole blood was nearly complete we found comparable increases in the corpuscle amino nitrogen by calculating this value from data obtained from the analysis of both laked and unlaked blood filtrates. The experimental data will be presented later.

The second argument which Simon presents which tends to show that the free amino acids of the corpuscles are not permitted

to diffuse out of the cell into the sulfate-tungstate diluting fluid in the preparation of an unlaked blood filtrate is simply that this extra amino nitrogen can be washed out by resuspending the corpuscles in a second portion of sulfate-tungstate solution. Simon found by this process of washing the cells, that the amino acid nitrogen content of the two washings was very nearly the same as that found in a whole blood filtrate prepared by laking the red corpuscles.

We have repeated these experiments also and in no case have we found the amino acid nitrogen content of such washings to be anywhere nearly as great as that found in a laked blood filtrate. On the contrary the values found were very nearly the same as those of the unlaked blood filtrate. Our data are given in Table III.

We can, therefore, see no justification in Simon's conclusion that in the preparation of the unlaked blood filtrate the free amino acids do not diffuse out into the diluting fluid to the extent characteristic of the blood. Further evidence based on the study of the distribution of amino acids between the corpuscles and plasma is presented supporting this view.

#### EXPERIMENTAL

*Preparation of Blood Filtrates*—The laked blood filtrates were prepared according to the well known Folin-Wu procedure.

The unlaked blood filtrates were prepared according to the directions published by Folin (4) in 1930 with the one exception that the precipitated proteins were filtered off instead of centrifuged. To be able to filter the precipitated proteins is a distinct advantage when a series of blood filtrates is prepared simultaneously. For this filtration it is necessary to use a good grade of filter paper,<sup>1</sup> folded (fluted) so as to expose a large filtering surface. It is essential that the tip of the folded paper should fit tightly into the stem of the funnel. Under these conditions filtration is rapid and a maximum yield of water-clear filtrate (37 to 38 cc. from 5 cc. of blood) is obtained in about 15 minutes. The filtration of

<sup>1</sup> We have found Schleicher and Schull No. 597 filter paper very satisfactory. Whatman No. 41 filter paper permits rapid filtration but we have found that this paper contains enough ammonia to become a disturbing factor in the amino acid nitrogen determination.

the unlaked blood should not be continued for more than 20 minutes, for on longer standing the red corpuscles begin to disintegrate due to the excess acid present. The filtering of the precipitated proteins from laked blood is not as rapid nor is the yield of filtrate as large, usually 25 to 28 cc. from 5 cc. of blood.

*Experiments on Addition of Amino Acids to Whole Blood in Vitro*—The amino acids were added to the whole blood by two methods: (1) by adding to the blood a small volume of an isotonic salt solution containing the equivalent of 10 mg. per cent of amino nitrogen, and (2) by adding a weighed amount of solid amino acid equivalent to 10 mg. per cent. Both methods gave equally good results.

1 cc. of the amino acid solution containing 10 mg. per cent of amino nitrogen or the weighed amount of solid amino acid was introduced into a 250 cc. beaker. Into a second 250 cc. beaker were measured 100 cc. of blood.<sup>2</sup> The blood was then poured rapidly but gently into the beaker containing the amino acid. After the blood was gently poured back and forth between the two beakers a number of times to insure complete mixing, it was poured into a 300 cc. Erlenmeyer flask. The flask was stoppered and placed in a warm water bath (37°) for 1 hour and gently shaken at intervals during this period.

The amino nitrogen content of the plasma and of the whole blood in both laked and unlaked blood filtrates was determined by the modified colorimetric method. From these data the content of amino nitrogen in the red corpuscles was calculated. Table II gives a summary of the values found.

It is seen from these values that in all but three cases the recovery of the added amino nitrogen was better than 96 per cent, the poorest was 91.7 per cent. The increase in the amino nitrogen content of the red corpuscles, calculated from both laked and unlaked blood filtrate analysis, is comparable in each experiment except one. In this case the amino nitrogen recovered in the

<sup>2</sup> Samples of sheep, dog, and chicken blood were used. The blood samples from sheep and chicken were obtained fresh from the abattoir and were generally about 45 minutes to an hour old before they reached the laboratory and work upon them started. The dog blood was obtained in large quantities from the femoral artery in conjunction with other experiments.

unlaked blood filtrate was only 91.7 per cent. If we assume complete recovery in this analysis, *i.e.* 14.43 mg. per cent instead of 13.24 mg. per cent, the calculated increase in the corpuscle amino nitrogen would be 7.45 mg. per cent while that of the laked cells is 7.5 mg. per cent.

From these experiments it appears that the explanation of Simon's results lies in the fact that complete recovery of the added amino nitrogen was not obtained.

*Experiments on "Washing Out" Extra Amino Nitrogen from Red Corpuscles*

A few experiments of this type were performed according to the description given by Simon; *i.e.*, with the use of two portions of sulfate-tungstate solution. However, our results did not conform to those reported by him. We, therefore, put the experiment to a more severe test, using three washings and increasing the time of contact of the cells with the diluting fluid from 5 minutes to 15 minutes.

Our procedure was as follows: 5 cc. of blood were added to 40 cc. of the regular sulfate-tungstate solution, mixed gently, and allowed to stand for 15 minutes. Without precipitating the proteins the mixture was centrifuged and the clear supernatant liquid was poured into an Erlenmeyer flask. The corpuscles were then resuspended in 25 cc. of 1.5 per cent sodium sulfate (anhydrous) solution and allowed to stand for 15 minutes. This mixture was centrifuged and the clear supernatant liquid added to the first washing. This process was repeated a third time. Sodium tungstate was omitted from the solution of the second and third washings so as to prevent the presence of an excess of this salt. To the combined washings, 5 cc. of  $\frac{1}{3}$  N sulfuric acid were added and the precipitated proteins filtered off. This filtrate is a 1:20 dilution of the original blood. The amino nitrogen was determined in this filtrate as well as in filtrates prepared from laked and unlaked blood. Table III gives the results of these analyses.

In not a single case does the amino nitrogen content of the washings approach the value found in the laked blood filtrate, but on the contrary it is nearly the same as that found in the unlaked blood filtrate.



TABLE II  
*Experiments Showing Recovery of Amino Acids Added to Blood*

Experiment No	Source of blood	Filtrate of laked or unlaked blood alone or with amino acid	NH <sub>2</sub> -N <sub>2</sub>			Corpuscles $\times$ 100 Plasma	Cell volume	Increase in corpuscular NH <sub>2</sub> -N <sub>2</sub>	Recovery of NH <sub>2</sub> -N <sub>2</sub>
			Whole blood	Plasma	Corpuscles				
			mg per cent	mg per cent	mg per cent		per cent	mg per cent	per cent
1	Sheep	Laked	6.7	4.96	7.64	154	65		
		+Amino acid	16.5	18.66	15.35	82.2		7.14	99
		Unlaked	4.1	4.96	3.63	73.3			
2	"	+Amino acid	13.9	18.66	11.35	60.5	54	7.47	98.6
		Laked	6.51	5.52	7.35	133			
		+Amino acid	16.23	18.0	14.7	81.8		7.35	98.4
3	"	Unlaked	3.58	5.52	1.93	35	64		
		+Amino acid	13.4	18.66	9.5	51		7.57	98.7
		Laked	8.68	5.95	10.2	172			
4	Dog	+Amino acid	17.3	19.0	16.35	86	54	6.15	92.6
		Unlaked	3.92	5.95	2.78	46.8			
		+Amino acid	13.7	19.0	10.70	56.4		7.92	98.4
5	"	Laked	7.82	4.69	10.5	224	34		
		+Amino acid	17.25	16.25	18.1	111.3		7.6	96.7
		Unlaked	4.23	4.69	3.84	82			
6	Sheep	+Amino acid	14.05	16.25	12.3	75.7	52	8.46	98.7
		Laked	6.99	4.76	11.3	237			
		+Amino acid	17.0	16.1	18.8	117		7.5	100
7	Dog	Unlaked	4.43	4.76	3.8	80	51		
		+Amino acid	13.24	16.1	7.76	48.2		3.9	91.7
		Laked	7.37	5.15	9.42	180			
8	"	+Amino acid	16.2	18.4	14.15	76	36	4.73	93.2
		Unlaked	3.76	5.15	2.48	48.2			
		+Amino acid	13.75	18.4	9.45	51.4		6.97	100
9	"	Laked	8.24	5.13	11.2	218	53		
		+Amino acid	18.13	17.84	18.4	103		7.2	99.5
		Unlaked	4.25	5.13	3.41	66.5			
10	"	+Amino acid	14.77	17.84	11.8	66.2	36	8.39	103
		Laked	7.45	5.19	11.5	222			
		+Amino acid	17.08	17.72	15.9	89.7		4.4	97.8
11	"	Unlaked	4.6	5.19	3.56	68.6	53		
		+Amino acid	14.6	17.72	9.04	51		5.48	100
		Laked	9.27	6.96	11.3	162			
12	"	+Amino acid	19.76	20.1	19.8	98.5	53	8.5	102.5
		Unlaked	5.53	6.96	4.27	61.5			
		+Amino acid	15.5	20.1	11.4	56.7		7.13	100

TABLE II—*Concluded*

Experiment No	Source of blood	Filtrate of laked or unlaked blood alone or with amino acid	NH <sub>2</sub> -N <sub>2</sub>			Corpuscles × 100 Plasma	Cell volume	Increase in corpuscle NH <sub>2</sub> -N <sub>2</sub>	Recovery of NH <sub>2</sub> -N <sub>2</sub>
			Whole blood	Plasma	Corpuscles				
			mg per cent	mg per cent	mg per cent				
10	Chicken	Laked	16 65	9 3	29 8	321	36		
		+Amino acid	26 6	24 9	29 8	119 5		0 0	100
		Unlaked	6 86	9 3	2 52	27 1			
11	"	+Amino acid	17 0	24 9	3 06	13 2	36	0 76	101
		Laked	16 65	9 3	29 8	321			
		+Amino acid	27 4	24 0	33 3	138 5		3 5	102 5
		Unlaked	6 86	9 3	2 52	27 1			
		+Amino acid	17 3	24 0	5 28	22 0		2 76	104

In all the above experiments except Experiment 11, 10 mg per cent of amino nitrogen as glycine were added 10 mg per cent of amino nitrogen as glutamic acid were added in Experiment 11

TABLE III

*Showing That "Extra" Amino Nitrogen in Red Corpuscles Cannot be Washed Out*

Experiment No	NH <sub>2</sub> -N <sub>2</sub>			Source of blood
	Normal unlaked blood	Three combined washings	Laked blood	
	mg per cent	mg per cent	mg per cent	
1	3 54	3 62	7 4	Dog
2	3 76	3 8	7 37	"
3	5 5	5 77	8 52	"
4	5 2	5 0	8 65	"
5	4 2	4 7	7 7	"
6	4 23	4 73	7 84	"
7	6 86	8 7	16 65	Chicken
8	6 86	8 44	16 65	"

### *Experiments on Distribution of Amino Acid in Blood between Red Corpuscles and Plasma*

If the amino acids present in the blood stream are capable of diffusing through the red blood cell wall and thereby establishing

themselves in equilibrium between the corpuscle and plasma, one should expect that amino acids added to whole blood would distribute themselves between the plasma and corpuscles to the same extent as that in the original blood. On reviewing the literature dealing with this type of experiment we find that this is not the case when the amino acid nitrogen is determined on a filtrate prepared from laked whole blood. Invariably the distribution ratio decreases as the amino acid nitrogen content of the whole blood increases, no matter how the amino acid level in the whole blood is raised, that is, by *in vitro* addition experiments, by injection of amino acids into the blood stream of a normal animal, or by rapid absorption of the products of protein digestion from the alimentary tract.

We have performed a number of experiments with the purpose of studying the distribution ratio of amino acid nitrogen between the corpuscles and plasma of whole blood. The whole blood amino nitrogen values were determined on both laked and unlaked blood filtrates. The corpuscle amino nitrogen content was calculated with both sets of data. From the corpuscle and plasma amino nitrogen values the distribution ratio was calculated and is expressed as the result of the following formula

$$\frac{\text{Corpuscle NH}_2\text{-N, mg per cent}}{\text{Plasma NH}_2\text{-N, mg per cent}} \times 100$$

*In Vitro Addition Experiments*—The distribution ratio of the amino nitrogen between whole blood and plasma found in the *in vitro* addition experiments are included in Table II. From the inspection of this table it is seen that the distribution ratio based on the analysis of laked whole blood filtrates invariably decreases to about one-half of its original value by the addition of 10 mg per cent of amino acid nitrogen. On the other hand the distribution ratio based on the analysis of unlaked whole blood filtrates remains very nearly the same as the original value after the addition of 10 mg per cent of amino acid nitrogen. The two distribution ratios found experimentally by the analysis of unlaked blood filtrates before and after the addition of amino acid to whole blood can scarcely be expected to be identical even if they are actually so, due to the fact that an error, occurring in the determination of the amino acid nitrogen content in any of the filtrates, will not only be

included in the calculated distribution ratio but will also be magnified in this calculation. Therefore, we consider that the results here reported show definitely that amino acids added to whole blood *in vitro* distribute themselves between the corpuscles and the plasma to the same extent as that characteristic of the normal blood sample.

*Intravenous Injection of Amino Acids into Dogs*—In order to simulate physiological conditions more closely than is possible in *in vitro* experiments, we have injected amino acids dissolved in isotonic salt solution, directly into the blood stream.

In Table IV are recorded the results of three such experiments. In Experiments 1 and 2, 2.5 gm. of glycine dissolved in 25 cc. of

TABLE IV  
*Intravenous Injection of Amino Acids*

Experiment No	Time after injection	Cell volume	Whole blood $\text{NH}_4\text{-N}_2$		Plasma $\text{NH}_4\text{-N}_2$	Corpuscle $\text{NH}_4\text{-N}_2$		Corpuscle Plasma $\times 100$	
			Un-laked	Laked		Un-laked	Laked	Unlaked	Laked
	min	per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent		
1		45.5	4.75	7.1	5.88	3.4	8.57	57.8	145
	5		10.1	11.2	12.5	7.23	9.64	57.8	77
	15		6.64	9.05	8.68	4.2	9.48	48.5	109
2		33	4.2	7.7	4.97	2.61	13.2	52.6	266
	8		8.8	11.05	10.33	5.67	12.5	55.0	121
	30		5.88	9.1	7.61	2.36	12.1	31.0	159
3		36.6	3.75	7.0	4.34	2.73	11.60	63.0	267
	5	33	7.8	10.6	8.76	5.88	14.35	66.0	164
	15	34	5.44	8.64	6.4	3.59	13.0	56.0	203

isotonic salt solution were injected into a saphenous vein of each of two dogs weighing 18 kilos and 17.5 kilos respectively. In Experiment 3, 6 gm. of an amino acid mixture prepared from horse corpuscles, dissolved in 60 cc. of physiological salt solution, were injected into a saphenous vein of a dog weighing 23.5 kilos. This dog became very sick shortly after the injection was completed and died between 3 and 4 hours later. Samples of blood were drawn at various intervals of time from the opposite leg in the case of each dog. The amino acid nitrogen determinations on all filtrates from one experiment were made simultaneously.

Table IV shows that when the amino acid concentration in the blood stream is increased in this manner, the distribution ratio invariably decreases when calculated from laked blood analyses. It is of interest to note that as the amino nitrogen content of the whole blood decreases the distribution value again approaches its high value. On the other hand the distribution ratio, based on calculations made on data obtained from unlaked blood analyses, remains nearly constant.

*Absorption of Amino Acids from Alimentary Canal*—From the above experiments it could be expected that the same relationships between the distribution ratios could be found in the blood stream during normal absorption of amino acids from the alimentary tract. We have performed a number of experiments in which dogs that had fasted for a period of 24 to 48 hours were allowed to eat as much boiled horse meat as they desired. Blood samples taken before feeding and at various intervals of time after feeding were analyzed for amino acid nitrogen. The results were in every way similar to the *in vitro* addition experiments and the intravenous injection experiments. We shall, therefore, forego the presentation of the tabulated figures.

We interpret these results as showing that (1) the amino acids present in the blood stream as a foodstuff are freely diffusible into and out of the red blood cells to the extent characteristic of the species; (2) the red blood cells contain one or more substances having one or more free amino groups capable of reacting with our reagents and are not capable of diffusing out of the red blood cell. It is because of these compounds that the laked blood filtrate gives an apparently higher amino acid content for whole blood than does the unlaked blood filtrate. The presence of such substances will account for the change in distribution ratio depending upon the concentration of the amino acids in the blood as found when laked blood is studied; but of course, they would have no effect on this ratio when studied by the use of unlaked blood filtrates because they are not included in this analysis.

Folin and Berglund (6), in 1922, suggested that part of this high amino nitrogen found in the red corpuscles was accounted for by the histones present there. We would suggest further that in the mammalian corpuscles more than one-half of this non-diffusible amino nitrogen is in the form of glutathione. One nitrogen of re-

duced glutathione reacts quantitatively with our reagents for this determination.

The use of the unlaked blood filtrate furnishes a suitable and convenient method for the separation of these two fractions of amino nitrogen. It is manifestly undesirable to confuse the study of one fraction of these substances by including in its analysis all the amino nitrogen of the second fraction.

TABLE V

*Summary of Amino Acid Nitrogen Found in Whole Blood, Corpuscles, and Plasma of Normal Men and Animals*

Experiment No	Species	No of analyses	Whole blood $\text{NH}_2\text{-N}_2$		Plasma $\text{NH}_2\text{-N}_2$		Corpuscle $\text{NH}_2\text{-N}_2$	
			Average	Range	Average	Range	Average	Range
			mg per cent	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent
1	Normal young men (fasting)	29	3.0	2.3 - 3.73	4.84	4.00 - 5.65	1.04	0.34 - 2.19
2	Normal young women (fasting)	8	2.89	2.32 - 3.3	4.78	3.86 - 5.46	0.37	0.05 - 0.834
3	Dog	18	5.56	4.23 - 6.35	6.0	4.69 - 7.66	3.83	1.95 - 4.34
4	Cat	2		5.59 - 7.76		8.25	5.8	
5	Rabbit	2		6.35 - 10.6		8.0	2.5	
6	Calf	1		5.23		5.73		
7	Sheep	6	3.85	3.54 - 4.36	5.36	4.96 - 6.27		
8	Chicken	1		6.86		9.3		2.52
9	Pigeon	9	5.06	4.43 - 6.1	9.6	7.7 - 11.55	0.96	0.0 - 1.84

*Amino Acid Nitrogen Content of Normal Blood*

All the values for the amino acid nitrogen in whole blood published in the literature with the exception of several analyses reported in two papers (5, 7) are based on the analysis of laked blood filtrates and are therefore in general too high. For example, the average value for normal human blood based on the analysis of laked blood filtrates is about 6 mg. per cent while the average value based on the analysis of unlaked blood is 3 mg. per cent. It

therefore seems advisable to record a summary of the average values we have found in unclaked blood filtrates for both normal human and animal bloods.

In Table V are summarized the values we have found for whole blood and plasma and also the calculated corpuscle value.

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## THE EFFECT OF INSULIN ON AMINO ACID AND UREA NITROGEN IN LAKED AND UNLAKED BLOOD

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Luck, Morrison, and Wilbur (1) in 1928 showed that insulin injections in rabbits cause a drop in the amino acid content of blood. Later this work was confirmed by Daniels and Luck (2) in experiments on humans. Since hypoglycemia due to hydrazine sulfate (3) is accompanied by an increase rather than a decrease in blood amino acid and since insulin injections cause a decrease in amino acid excretion (4), they concluded that the lowering of blood amino acid by insulin is probably due neither to hypoglycemia nor to a change in the rate of excretion. Kiech and Luck (5) later demonstrated by means of whole animal analyses on rats that, following insulin injections, the decrease in amino nitrogen was approximately balanced by an increase in urea nitrogen and concluded that insulin accelerates the catabolism of amino acids without a compensatory increase in the regeneration of amino acids from tissue proteins.

In 1929 Kerr and Krikorian (6) in a study on the effect of insulin on the distribution of non-protein nitrogen of blood obtained results confirmatory of the work of Kiech and Luck. They did, however, find considerable variation in the urea nitrogen content, sometimes getting an actual decrease and sometimes an increase of as much as 10 times the decrease in amino nitrogen. This and the fact that while the corpuscular amino nitrogen was approximately 50 per cent higher than the serum amino nitrogen, the drop caused by insulin was not proportionate, seemed to us to warrant further study. Since the amino nitrogen values for corpuscles are higher than for serum, we reasoned that possibly a part of the corpuscular amino nitrogen is in some way held within the cell in a non-diffusible form and while being included in laked blood



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analyses would not be present in unlaked blood which would contain only diffusible amino acids. If it could be shown that insulin caused the same number of mg. drop in the amino acid content of both laked and unlaked blood it might be interpreted that insulin affects only the diffusible amino acids.

TABLE I  
*Amino Acid and Urea Nitrogen As Determined by Folin's Methods*  
The results are measured in mg. per 100 cc. of blood.

Rabbit No	Amino acid N						Urea N in laked blood		
	Laked blood			Unlaked blood					
	0 hrs	2 hrs	5 hrs	0 hrs	2 hrs	5 hrs	0 hrs	2 hrs	5 hrs
Insulin-injected rabbits									
2	10 5	-1 9	-1 8	6 8	-2 0	-1 2			
2	9 5	-2 4	-2 7	6 8	-2 6	-2 5	28 4	+0 2	-0 8
3	10 9	-2 3	-2 3	8 0	-2 0	-1 8			
3	10 0	-1 4	-2 3	6 6	-1 2	-0 8	54 3	-0 5	-0 5
3	10 3	-2 1	-2 1	7 1	-2 2	-2 9	41 7	-0 7	-0 4
6	9 8	-1 2	-2 1	6 5	-0 7	-0 8	19 8	+1 3	+0 8
6*	8 4	-1 6		5 6	-1 2		25 1	+0 6	
5	8 6	-2 5	-2 7	5 7	-1 7	-1 3	25 3	-3 8	-4 6
10	8 6	-2 2	-2 1	6 2	-1 3	-1 3	23 2	-1 1	+1 2
Average	9 6	-2 0	-2 3	6 6	-1 7	-1 6	31 1	-0 6	-0 7
Controls									
5	9 2	-1 1	-0 1	6 4	+0 1	+0 4	19 8	+1 2	+0 1
2	9 7	-0 2	-1 0	5 9	+0 1	-0 1	20 4	+6 6	+5 3
Average	9 5	-0 7	-0 0	6 2	+0 1	+0 2	20 1	+3 9	+2 7

\* Died in convulsions 3 hours after injection.

### EXPERIMENTAL

Male rabbits which had been fasted for 24 hours previous to the experiment were used. Blood was drawn by means of a dry oxalated syringe from the ear vein or in some cases where the vein was closed, from the heart. Three samples of 2.5 cc. each were drawn,

one just before the injection of insulin, one 2 hours after, and the last 5 hours after injection. The insulin was first diluted with water to contain 2 units per cc. and an amount equal to 1 unit per kilo of body weight was injected. In the controls normal saline solution of approximately the pH of the insulin solution was injected in place of the insulin. Both laked and unlaked filtrates

TABLE II

*Amino Acid and Urea Nitrogen As Determined by Van Slyke's Manometric Methods*

The results are measured in mg per 100 cc of blood.

Rabbit No	Amino acid N						Urea N					
	Laked blood			Unlaked blood			Laked blood			Unlaked blood		
	0 hrs	2 hrs	5 hrs	0 hrs	2 hrs	5 hrs	0 hrs	2 hrs	5 hrs	0 hrs	2 hrs	5 hrs
Insulin-injected rabbits												
2	8 8	-1 2	-1 5	3 9	-1 6	-1 6	23 9	+0 3	-2 6	22 9	-0 1	-0 4
10	10 4	-0 9	-1 8	4 6	-0 8	-1 8	21 7	-1 2	-2 0	19 5	+0 2	+1 8
14	12 1	-2 3	-1 8	4 6	-1 9	-2 1	22 5	+4 5	+6 9	19 7	+6 2	+7 6
15	11 1	-1 6	-2 4	6 3	-1 3	-2 0						
13*	11 0	-1 8	-2 4	5 4	-1 3	-2 0	28 1	+0 6	+2 1	23 6	-1 0	+2 1
Average	10 7	-1 6	-2 0	5 0	-1 4	-1 9	24 1	+1 5	+1 1	21 4	+1 3	+2 8
Controls												
2	12 1	-0 1	+0 1	4 5	+0 9	+0 8	25 5	+1 6	-1 5	25 9	-1 8	-1 9
10	13 0	+0 3	+0 3	6 8	+0 6	-0 2	17 4		-0 1	17 8	-0 8	-1 1
Average	12 6	+0 1	+0 2	5 7	+0 8	+0 3	21 5	+1 6	-0 8	21 9	-1 3	-1 5

\* Convulsions started 2 hours after injection. The rabbit died in convulsions shortly after last blood was drawn.

were made from each sample according to the methods of Folin, 1 cc. of blood being placed in a centrifuge tube, the appropriate reagents added (the final dilution being 10 cc. in each case), and the precipitated protein removed by centrifuging. The sugar was determined on a separate filtrate by Folin's ferricyanide method. This was done to check the activity of the insulin, but as the results

were typical and of no significance to this paper, they are not included in the tables.

In the first series of experiments as listed in Table I Folin's methods for amino acid and urea were used, 4 cc. of the filtrate for amino acid and 2 cc. for urea. Considerable difficulty, however, was often experienced in matching the colors in the amino acid determination, and for this reason we later repeated the work using Van Slyke's manometric methods, again using 4 cc. of the filtrate for amino acid and 2 cc. for urea. These results are given in Table II.

In both tables the initial amino acid and urea values are given in terms of mg. of nitrogen per 100 cc. of blood. The figures given for the 2 and 5 hour periods also are in terms of mg. of nitrogen per 100 cc. of blood and represent the change from the initial value, a rise being indicated by a plus sign and a fall by a minus sign.

#### DISCUSSION

By an inspection of the urea nitrogen values listed in Tables I and II it will be seen that while we obtained substantially the same variations as did Kerr and Krikorian, our averages do not confirm their findings of a rise in blood urea following insulin injection. In fact if we were to average all of our results before and after insulin and before and after normal saline as they did with their results we would find an increase of 0.7 mg. of urea nitrogen following normal saline and 0.6 mg. following insulin. In the cases of insulin-injected Rabbit 5 and control Rabbit 2 in Table I and Rabbit 14 in Table II where the change from the initial urea value is so much greater than in the other rabbits we wonder if some other factor may not have entered, and doubt if these should be included in a general average. If these are eliminated, the general average shows a drop of 0.1 mg. of urea nitrogen following insulin and a drop of 0.5 mg. following normal saline. However, the fact that no change in blood urea nitrogen can be demonstrated after insulin injection might be accounted for by an increased excretion and does not argue against an increased formation as was demonstrated by the work of Kiech and Luck.

Folin's method gave substantially the same decreases in amino nitrogen values after insulin injections in both laked and unlaked

blood. Difficulty was experienced in matching colors in most of the unlaked samples and in some of the laked samples which may account for the variations. In Van Slyke's method this factor is eliminated and we find a very close agreement between the decreases in amino nitrogen in laked and unlaked blood. Thus one may conclude that only the diffusible amino acid is affected by insulin injections.

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## THE RING STRUCTURE OF URIDINE

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(Received for publication, May 26, 1933)

The furanoside ring structure of the sugar portion of the two purine nucleosides, adenosine<sup>1</sup> and guanosine,<sup>2</sup> has recently been established by us by the application of direct chemical methods. The work was later extended to the elucidation of the structure of the two pyrimidine ribosides.

Since, by deaminization, cytidine is readily transformed into uridine,<sup>3</sup> having properties identical with those of the uridine from uridylic acid, it would follow that the sugar portions of these two nucleosides have the same ring structure. In the present investigation the ring structure of uridine was studied, inasmuch as this nucleoside is more accessible in large quantities.

Uridine is characterized by its resistance to hydrolysis by dilute mineral acids,<sup>3</sup> but this is no indication of the presence of a pyranoside ring structure since dihydrouridine,<sup>4</sup> obtained by catalytic reduction of uridine with hydrogen, is readily hydrolyzed by heating for 90 minutes with 3 per cent sulfuric acid. It seems evident that the stability of uridine is dependent on the double bond in the uracil portion of the molecule.

Several methods of attacking the problem appeared promising. By a modification of the procedure in the isolation of the acetyluridine a fully acetylated (triacetyl) uridine has now been obtained, whereas in the earlier work of Levene and Jacobs,<sup>3</sup> difficulty was encountered in the preparation of this derivative.

<sup>1</sup> Levene, P. A., and Tipson, R. S., *Science*, **74**, 521 (1931); *J. Biol. Chem.*, **94**, 809 (1931-32).

<sup>2</sup> Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **97**, 491 (1932).

<sup>3</sup> Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, **43**, 3150 (1910).

<sup>4</sup> Levene, P. A., and La Forge, F. B., *Ber. chem. Ges.*, **45**, 608 (1912).

Simultaneous deacetylation and methylation of triacetyl uridine gave a methylated uridine which retained the stability towards hydrolysis by acids which is characteristic of the parent substance. The product was invariably colored yellow or pale brown and consequently, on catalytic hydrogenation, the extent of reduction could not be judged by testing samples with dilute bromine water.

The simultaneous hydrolysis and oxidation of methylated uridine with concentrated nitric acid, with a view to the isolation and identification of the methylated dibasic acid from the sugar portion of the molecule, was apparently complicated by the formation of a product analogous to that obtained by the action of the same reagent on unsubstituted uridine.<sup>4</sup>

The simultaneous hydrolysis and oxidation of trimethyl uridine with hydrobromic acid and bromine under the conditions previously utilized for cytidine<sup>4</sup> gave but a small yield of methylated ribonolactone, due presumably to the difficulty of hydrolysis of the methylated nucleoside and the subsequent decomposition of the scission product by the mineral acid.

By catalytic reduction of uridine acetate with hydrogen, followed by simultaneous deacetylation and methylation of the product, a methylated dihydrouridine was obtained. This was much more readily hydrolyzed by mineral acids than was methylated uridine. We were not, however, able to isolate the methylated ribose in a state of sufficient purity for comparison with the trimethyl ribofuranose from methylated adenosine<sup>1</sup> and methylated guanosine.<sup>2</sup>

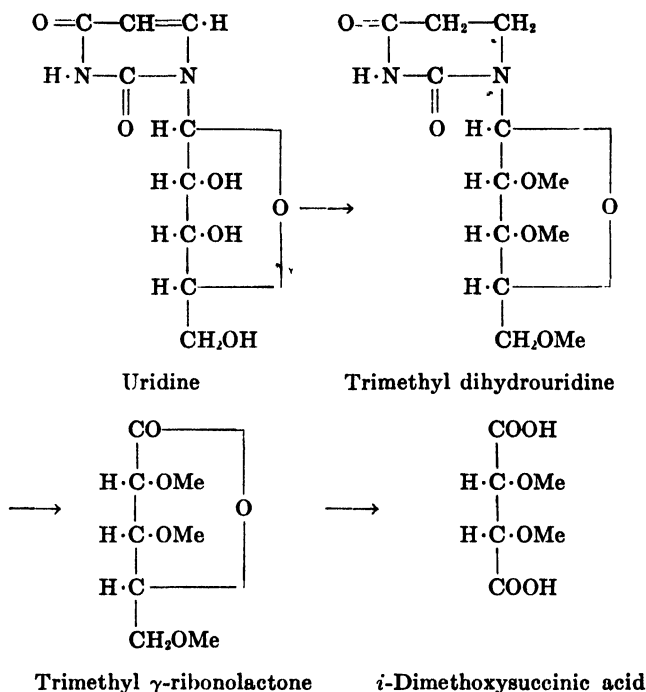
Simultaneous hydrolysis and oxidation of methylated dihydrouridine, with hydrobromic acid and bromine water, gave a trimethyl-ribonolactone which was identified as the  $\gamma$ -lactone inasmuch as on oxidation with nitric acid it formed *i*-dimethoxysuccinic acid under conditions which lead to the formation of trimethoxyglutaric acid from trimethyl  $\delta$ -ribonolactone.<sup>5</sup> It follows that uridine is a ribofuranoside.

Nearly a year after our publications on the ring structure of the ribose residue of adenosine and guanosine, Brederick<sup>6</sup> published an article on the same subject. The author arrived at a conclusion identical with ours on the basis of the preparation of a

<sup>4</sup> Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **93**, 623 (1931).

<sup>6</sup> Brederick, H., *Ber. chem. Ges.*, **66**, 198 (1933)

trityl derivative of the nucleosides, but Brederick's reference to our work was very casual. We wish to emphasize, however, that in the case of pentosides, the trityl reaction cannot be regarded as very rigid evidence of structure (as was first demonstrated by Hockett and Hudson<sup>7</sup>) and indeed, it appears probable that the crude "trityl" derivative of uridine described by Brederick<sup>8</sup> is a mixture of a mono- and a disubstituted nucleoside. For this reason we continued our work, even after Brederick's publication dealing with the problem of uridine, and have succeeded in isolating monotrityluridine in pure crystalline form.



#### EXPERIMENTAL

*Preparation of Triacetyl Uridine*—10 gm. of uridine were acetylated with 130 cc. of acetic anhydride containing 0.25 gm. of fused

<sup>7</sup> Hockett, R. C., and Hudson, C. S., *J. Am. Chem. Soc.*, **53**, 4456 (1932).

<sup>8</sup> Brederick, H., *Ber. chem. Ges.*, **65**, 1830 (1932).



sodium acetate and the product isolated as described for triacetyl guanosine.<sup>2</sup> The uridine acetate was obtained as a pale yellow, flaky, glass-like solid. Yield 15 gm. It was insoluble in cold or hot petroleum ether, ether, or toluene, but soluble in the following solvents in the cold: chloroform, glacial acetic acid, pyridine, acetone, methyl alcohol, ethyl alcohol, ethyl acetate, and water.

The substance had the following composition

5.115 mg. substance:	9.145 mg. CO <sub>2</sub> and 2.300 mg. H <sub>2</sub> O
100 " "	required 5.28 cc. 0.1 N HCl (Kjeldahl)
C <sub>11</sub> H <sub>13</sub> O <sub>6</sub> N <sub>2</sub> .	Calculated. C 48.63, H 4.9, N 7.57
	Found. " 48.75, " 5.0, " 7.39
100 mg. substance required	(a) 8.11 cc. 0.1 N NaOH (alkaline hydrolysis)
	(b) 8.07 " 0.1 " " " "
C <sub>9</sub> H <sub>9</sub> O <sub>6</sub> N <sub>2</sub> (COCH <sub>3</sub> ) <sub>3</sub> .	Calculated. COCH <sub>3</sub> 34.86
	Found. " (a) 34.88, (b) 34.72

*Hydrogenation of Uridine Acetate*—The acetate from 10 gm. of uridine was dissolved in 50 cc. of ethyl acetate, 0.25 gm. of Adams' catalyst<sup>9</sup> was added, and the suspension was shaken with hydrogen at a pressure of 45 pounds per sq. inch. The mixture was shaken during 48 hours, a little catalyst being added from time to time. It was then filtered and the catalyst well washed with acetone. The combined filtrate and washings were evaporated to dryness under diminished pressure, giving a pale yellow glass. Yield 15 gm. In some cases the reduction was not complete and the above treatment was repeated until a sample of the product readily reduced boiling Fehling's solution after hydrolysis with dilute hydrochloric acid, and gave only a faint decolorization of dilute bromine water.

The triacetyl dihydrouridine was insoluble in petroleum ether and in toluene but dissolved in acetone, ethyl acetate, glacial acetic acid, methyl alcohol, chloroform, and hot water.

*Methylation of Dihydrouridine*—15 gm. of triacetyl dihydrouridine were dissolved in 300 cc. of acetone and treated with 298 cc. of 30 per cent sodium hydroxide solution and 147 cc. of dimethyl sulfate as previously described for the methylation of adenosine.<sup>1</sup>

The product was a pale brown gum, soluble in chloroform, ben-

<sup>9</sup> Adams, R., Voorhees, B., and Shriner, R. L., in Gilman, H., *Organic syntheses*, New York, coll. 1, 452 (1932).

zene, acetone, methyl alcohol, and water but insoluble in dry ether or petroleum ether. Yield 9.5 gm.

*Simultaneous Hydrolysis and Oxidation of Methylated Dihydrouridine*—16 gm. of twice methylated dihydrouridine were dissolved in 200 cc. of 3 per cent aqueous hydrobromic acid. 13 cc. of bromine were now added, in portions of 1 cc. at intervals of 30 minutes, during 7 hours at 85°. The mixture was then kept at room temperature overnight, after which some insoluble gummy material was filtered off. The filtrate was freed of excess bromine by aeration, silver carbonate was added until all the mineral acid had been neutralized, the mixture was filtered, and the silver salts well washed with water.

The combined filtrate and washings were saturated with hydrogen sulfide, the excess of which was then removed by aeration. The silver sulfide was filtered off and the clear filtrate evaporated under diminished pressure to a yellow-brown gum containing some crystalline material. This product was extracted several times with chloroform, some of the gum remaining undissolved. The chloroform solution was evaporated to a syrupy gum. Weight 7.5 gm. This material was dissolved in 20 cc. of chloroform and slowly dropped into 500 cc. of dry ether with shaking, whereby some insoluble material was precipitated. This was filtered off and the filtrate evaporated under diminished pressure to a syrup. Weight 6 gm. This was repeatedly lixiviated with dry ether and the ether extract evaporated under diminished pressure to a syrup. Weight 5 gm.

The ether-soluble product was now boiled for 5 hours with 150 cc. of methyl alcohol containing 1.5 per cent of hydrogen chloride. After the mixture had been cooled and the acid neutralized with dry silver carbonate, the methyl alcohol was removed under diminished pressure and the resulting syrup dissolved in dry ether, filtered from a little insoluble gum, and the filtrate evaporated to a syrup. Weight 4.5 gm. The substance was purified by distillation under high vacuum. A colorless syrupy product was collected at 85–87° at 0.05 mm. (bath temperature 100–104°). Yield 3.7 gm.  $n_D^{22} = 1.4495$ . Analysis showed it to consist of a mixture of trimethyl ribonolactone with its methyl ester.

1 gm. of this substance was hydrolyzed with 25 cc. of 4 per cent hydrochloric acid in a water bath at 85° during 2.5 hours. The

lactone was isolated in the usual way and distilled under high vacuum. It boiled at 90–95° at 0.05 mm. (bath temperature 115–120°). The course of the hydrolysis of the lactone in aqueous solution was studied polarimetrically, the rotation still being negative after 120 hours. This has previously been found to be the case with trimethyl  $\gamma$ -ribonolactone,<sup>1</sup> whereas with trimethyl  $\delta$ -ribonolactone<sup>5</sup> the rotation becomes positive before 20 hours have elapsed.

Oxidation with concentrated nitric acid was effected in the usual manner and gave crystalline *i*-dimethoxysuccinic acid which yielded the characteristic methyl ester; m.p. 68°.

## FLUCTUATIONS OF THE BLOOD SUGAR IN VITRO\*

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(Received for publication, May 19, 1933)

In 1918 one of us (1) reported a noteworthy difference in the rate of dialysis of sugar from the blood of dogs, depending upon whether one used diabetic blood or normal blood to which glucose had been added. The diabetic blood sugar dialyzed irregularly, with a delay or complete interruption during one or more periods, usually the 2nd hour. The control, consisting of normal blood with added glucose, dialyzed at a regular rate, i.e. without any interruptions. Three hypotheses were presented as possible explanations: (1) that the retardation might be due to a clogging of the membrane by the increased fat or other lipid of the diabetic blood, (2) that new sugar was being formed during the dialysis of the diabetic blood, and (3) that part of the sugar of diabetic blood was in a combined colloidal state. The third hypothesis was believed to be the most plausible, but there was some evidence that the second, namely a new formation of sugar, might play a subordinate, if not the major, rôle.

Naturally the question whether the same phenomenon could be demonstrated in human blood was an important one. It was found (2) that human diabetic blood sugar dialyzed very irregularly, showing frequent delays in the fall of the sugar, and often even rises. However, controls of normal human blood, to which glucose had been added, exhibited similar irregularities, although perhaps not quite as marked as the diabetic blood. In these experiments hirudin or novirudin was used to prevent the blood from clotting, and either the Folin-Wu method (3) or the Folin modification of that method (4) was employed for analysis. The

\* Aided by a grant from the Lucius N. Littauer Foundation, Inc.

increases in the sugar content frequently exceeded 20 mg. per 100 cc., and since dialysis was occurring and hence some glucose was being lost, the figures may be considered minimal.

In some of the experiments mentioned above there occurred particularly marked fluctuations in the sugar content of dialyzing blood during the early stages of dialysis. This was most striking in experiments on diabetic blood, both from human subjects and from depancreatized dogs, but it was also observed when normal blood which had been fortified with glucose was used. It seemed as if dialysis for a short time had caused some change in physical conditions favorable either to glycolysis or to glucogenesis. The question arose, would this change, this unstable state due to a short dialysis, continue in evidence after the dialysis process was discontinued? Consequently, we have conducted experiments in which the blood was dialyzed, under conditions similar to those heretofore described, but for only a short time, after which it was transferred to a glass container. The blood sugar level throughout the experiment was determined by taking samples at intervals of about 15 or 20 minutes.

### *Method*

The blood, obtained from human subjects<sup>1</sup> or from dogs, by needle and syringe, was prevented from clotting by the use of either hirudin or novirudin, as a rule. In some instances, other anticoagulants, such as oxalate or fluoride, were used. Dialysis was conducted in the same manner as described in previous papers (1, 2, 5). At the end of the short dialysis (usually 15 minutes) the bag was removed from the bath and the blood transferred to a dry Pyrex flask and kept, stoppered, at room temperature. Samples of blood were taken for sugar determination before and at the end of dialysis, and at short intervals thereafter. In the figures, the time shown represents the period which elapsed from the time the blood was drawn.

In the first trials blood sugar was determined by the Folin modification of the Folin-Wu method<sup>2</sup> (4) or by the Myers-Bailey

<sup>1</sup> We wish to thank Drs. P. J. R. Schmahl, Sydney Gubin, Harry Leinoff, and other members of the staffs of Flower Hospital and the Metropolitan Hospital for aiding us in obtaining samples of blood.

<sup>2</sup> These experiments were performed by Dr. Marion Bell.

modification of Lewis and Benedict's method (6). Since these experiments gave results which were quite surprising, it was considered advisable to repeat the work, a method which would permit greater accuracy being used. The Shaffer-Somogyi method<sup>3</sup> (7) was adopted, since it has been shown to be very accurate and it is claimed that it includes a minimum of the non-sugar reducing bodies. Moreover, it is a titration method and, therefore, the personal factor is also minimized. In our hands this method yielded good results, as a rule. Later, still another procedure, which gave admirable results, was adopted. This was the analysis of an aliquot portion of a Somogyi filtrate (8) by the Hagedorn-Jensen method (9).

By all four methods the same phenomenon is frequently observed.<sup>4</sup> A brief period of dialysis of blood, either diabetic or normal blood fortified with glucose, is followed by a marked fluctuation in the blood sugar level. Sometimes drops of 40 to 60 mg. per 100 cc., followed by similar increases are noted. These irregularities sometimes occur immediately after dialysis; sometimes they are postponed for 2 or 3 hours; while occasionally they take place during the dialysis, as evidenced by a slight rise or a very sharp fall. Sometimes no fluctuations whatever occur. The irregularity in time made it impossible for us to study the phenomenon as fully as we had desired. We have done many experiments, in which the conditions were varied slightly, in the hope of finding some way of producing a peak or a low point at some fairly definite interval after dialysis in order to subject the blood to more searching analysis at that stage, but were not successful. Figs. 1 and 2 include typical examples, showing marked fluctuations, up and down, in 15 to 30 minute periods, of the blood sugar of various types of hyperglycemic blood. These fluctuations in many of the curves shown range from 30 to 70 mg. The conditions and methods of analysis used in the experiments illustrated vary as stated above, but it is impossible to give more than a few in the space available.

<sup>3</sup> We are indebted to Dr. Shaffer for the details of these methods in advance of publication.

<sup>4</sup> Preliminary references to this work were included in the report of Kleiner and Bell (10) presented before The Thirteenth International Physiological Congress, August 22, 1929, and of Kleiner (11), in 1930.

*Fluctuation of Blood Sugar in Undialyzed Blood*—During the course of this work the question of controlling these experiments by observing the blood sugar in undialyzed blood was taken up.

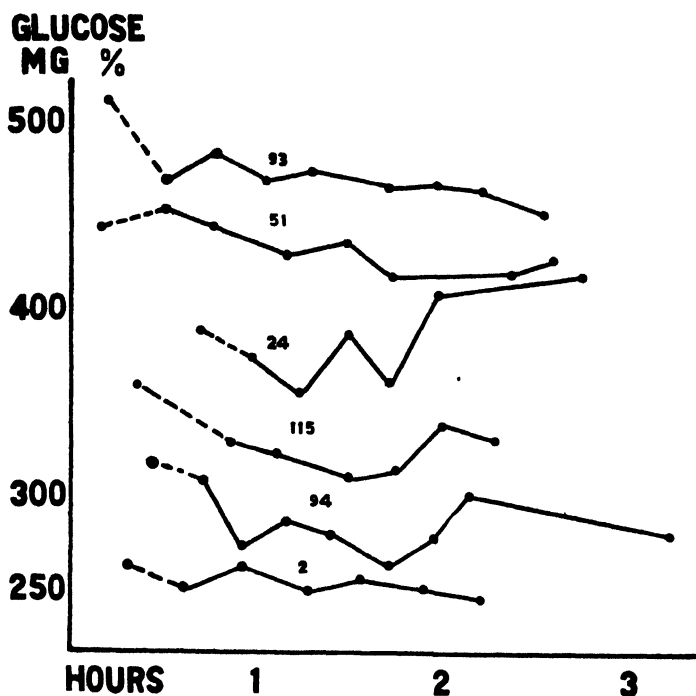


FIG. 1. Diabetic blood was dialyzed for a short time against Ringer's solution containing glucose and then transferred to a glass vessel. The broken line indicates the dialyzing period, the solid line the period in glass. Experiments 2 and 24 were on human blood, the others on dog blood. Ringer's solution contained 0.2 per cent glucose in Experiment 93, 0.3 per cent in Experiments 51 and 115, and 0.1 per cent in the others. Anticoagulant used: Experiments 51 and 93, novirudin; hirudin in the others. Analytical method used: Experiments 2, 115, 51, Shaffer-Somogyi; Experiment 94, Myers-Bailey; Experiment 24, Folin-Wu; Experiment 93, Somogyi-Hagedorn-Jensen. The experiments were carried out at room temperature.

This, at first glance, would seem to be unnecessary, since so many investigations of glycolysis have been reported and have indicated that the glycolytic curve is a slow gradual one. The rate of glycol-

ysis varies, to be sure, but no marked fluctuations such as we have found in dialyzing or in dialyzed blood have been noted in the

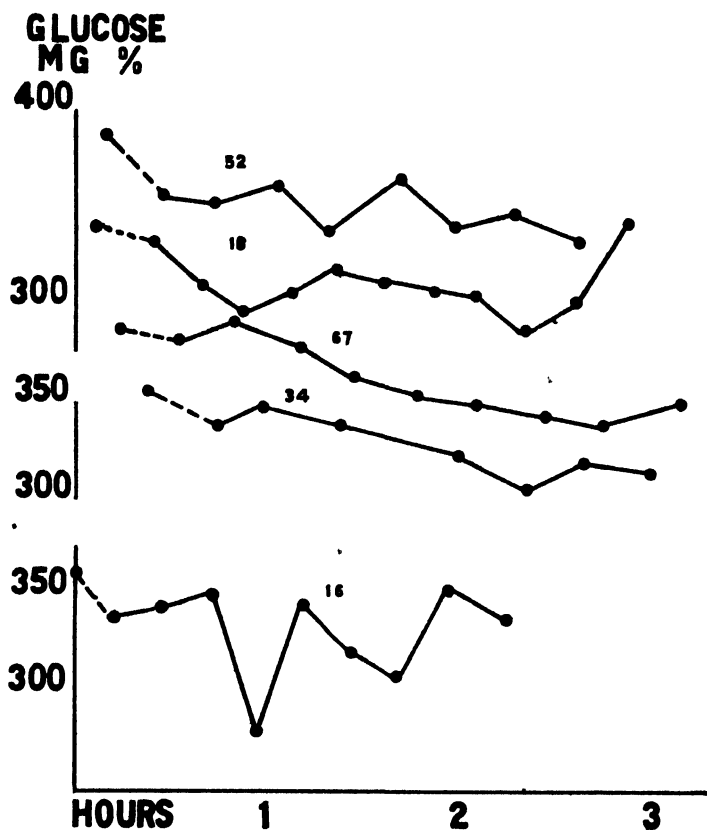


FIG. 2. Hyperglycemic blood dialyzed for a short time against Ringer's solution containing glucose. The broken line indicates the dialyzing period, the solid line the period in glass. Experiments 16 and 18 were on human diabetic blood; Experiment 52, on dog diabetic blood; Experiment 67, on normal dog blood with added glucose; Experiment 34, on normal human blood with added glucose. Ringer's solution contained 0.1 per cent glucose in Experiments 16, 34, and 18; 0.3 per cent glucose in Experiments 67 and 52. Novirudin was used in all. The Shaffer-Somogyi method was used in all. The experiments were carried out at room temperature.

literature. In the past, however, samples for analysis have been taken at long intervals, usually not shorter than 1 hour and, indeed,



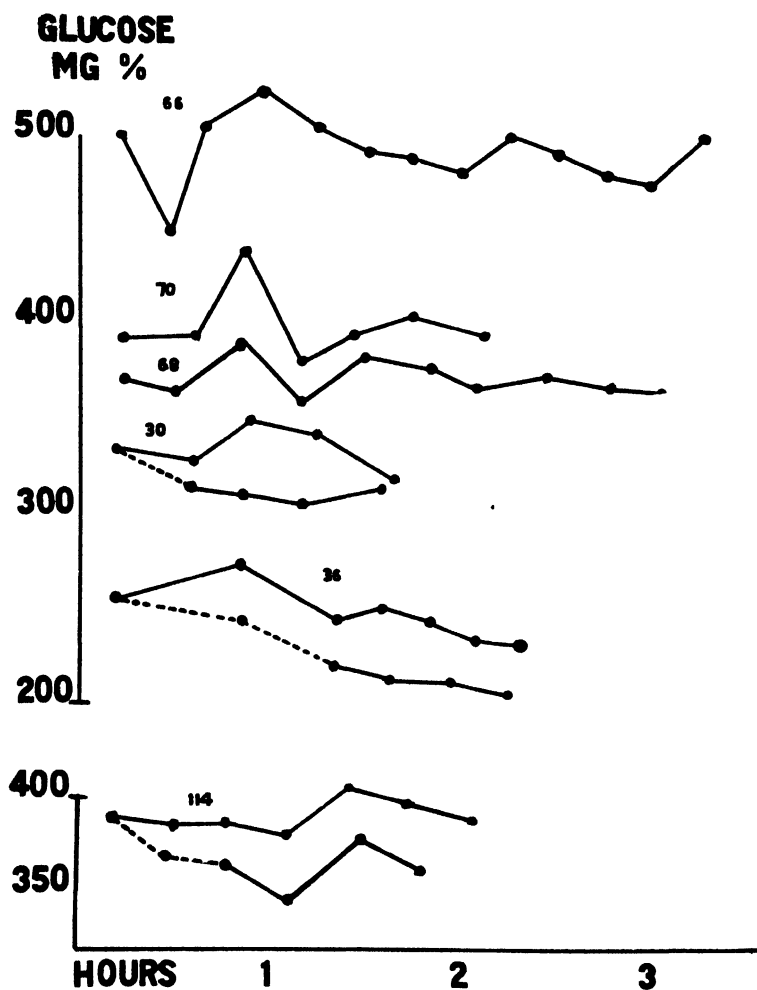


FIG. 3. Undialyzed hyperglycemic blood was kept at room temperature. In Experiments 30, 36, and 114, part of the blood was dialyzed for a short time (indicated by the broken line) and then transferred to a glass vessel. Experiment 36 was on normal dog blood with added glucose; Experiment 30, on normal human blood with added glucose; the remainder, on diabetic dog blood. The dialysis in Experiment 30 was against 0.1 per cent glucose in Ringer's solution, Experiment 36 against 0.2 per cent, and Experiment 114 against 0.3 per cent. Hirudin was used in Experiment 114, novirudin in all the others. The Somogyi-Hagedorn-Jensen method was used in Experiment 114, the Shaffer-Somogyi method in all the others.

much of the work heretofore described is based on 18 or 24 hour periods. We have, therefore, done a large number of simple glycolysis experiments, sampling at short intervals, and find that very frequently the glycolytic curve is a most irregular one. Some of these are shown in Fig. 3.

Although a preliminary short dialysis may initiate these fluctuations, this is not necessarily the case. In Fig. 3 we have represented some experiments which show two samples of the same blood, one dialyzed for a short time and then transferred to a glass

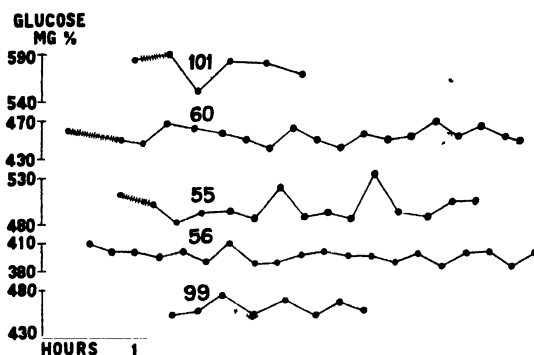


FIG. 4. Experiments with plasma. Experiment 60 was on normal dog plasma plus glucose, the others on plasma from diabetic dog blood. Novirudin was used in Experiments 55, 56, and 99; hirudin in Experiment 101; NaF in Experiment 60. The dialysis (indicated by cross-barred lines) was against Ringer's solution containing 0.3 per cent glucose in Experiments 55, 60, and 101; there was no dialysis in the other two. Thymol was added to the plasma in Experiments 55, 56, and 60. The Shaffer-Somogyi method was used in Experiments 55, 56, and 60; the Somogyi-Hagedorn-Jensen method in Experiments 99 and 101 on duplicate samples.

vessel, and the other undialyzed, kept in a glass vessel, and samples taken simultaneously from both. Experiment 114 shows a remarkable parallelism between the two curves; in this experiment duplicate samples of blood were analyzed at each point. In some cases the curve of the dialyzed blood is more irregular than that of the undialyzed, and in others the reverse is true.

We have performed a small number of experiments with plasma and can report results similar to those of whole blood. Examples are given in Fig. 4. It will be noticed that there is no downward

trend of the curves, no glycolysis in the ordinary sense of the term, as was the case with whole blood. This is in agreement with the earlier work of many. There are, however, marked fluctuations in most of the curves shown. This was not always found, and we were unsuccessful in discovering a procedure which would give us more or less standardized results. Although there are not enough experiments upon which to base a conclusion, it is our impression that a preliminary short dialysis of diabetic blood plasma yields greater fluctuations in the curve than are observed if there is no dialysis or if normal plasma, fortified with glucose, is used.

In a number of the experiments thymol was added to inhibit bacterial growth, and in others aseptic precautions were adopted. Bacteriological examination<sup>5</sup> at the end of these experiments indicated that microorganisms played no rôle in this phenomenon, since no relation could be found between the presence or absence of bacteria and the degree of fluctuations of the sugar. In this connection it may be mentioned that Tolstoi (12) states that bacterial contamination did not affect his results on glycolysis during a period of 24 hours.

#### DISCUSSION

The question of method may be answered by stating that fluctuations may in part be due to analytical errors—particularly in those experiments in which colorimetry was employed. But this cannot account for all the marked fluctuations described. Moreover, we have found the same type of thing with four different methods, two colorimetric and two titrimetric, performed by three different analysts. In a number of the experiments, in which marked fluctuations occurred, the accuracy of the results was assured by the analysis of duplicate samples of blood at every point in the curve.

If the phenomenon described could possibly be due entirely to experimental error, the inadequacy of the usual laboratory procedures for blood sugar is evident. In any case high blood sugar figures should not be expressed to the third decimal place, since the second either is doubtful or is likely to undergo serious fluctua-

<sup>5</sup> Our thanks are due Drs. Laura Florence and Margaret Hotchkiss, and Miss Miriam Diehl for making these bacteriological checks.

tions. In this connection we may say that the phenomenon has been observed (although not constantly) in blood samples taken into citrate, fluoride, or oxalate, respectively, as well as hirudin or novirudin; duplicate samples, treated with different anticoagulants, do not give parallel blood sugar curves along the entire course. We are convinced that the irregularity of the blood sugar curve *in vitro*, is due not entirely to analytical error but to some unknown change in the reducing substances of the blood.

In discussing this phenomenon—and probably the fluctuations in dialyzing, dialyzed, and undialyzed blood are all evidences of the same phenomenon—it must first be emphasized that the work has been done at room temperature. Lépine and Barral (13) have stated that blood, kept at 58°, showed marked increases in sugar content during short periods. This *sucre virtuel* could not be found by Macleod (14) but Lépine (15) explained that Macleod's experiments were performed at room temperature and the higher degree of heat was necessary in order to inhibit glycolysis. He asserts that *sucre virtuel* is the result of enzyme activity upon some substance having a glucoside linkage. The enzyme is found in the wall of blood vessels (16). Gabbe (17) claims to have confirmed Lépine's results, since he found that taka-diastase and emulsin increased blood sugar. We wish to emphasize, however, that in our experiments not only were increases seen, but also decreases, and these were just as great and just as rapid. The phenomenon which we have described is independent of glycolysis. It may occur during glycolysis, as in most of the experiments shown; or in the absence of glycolysis, as in the experiments upon plasma and upon blood treated with fluoride. Whether this phenomenon is the same thing as Dische (18) describes, we cannot say. By two new color tests he finds evidence in human blood of two hexoses besides glucose, one an aldose and one a ketose. These he designates *Nebenzucker*. In the incubator at 37° this vanishes in 2 hours. However, Dische states that at room temperature the ketose fraction *increases* in amount.

Myers and Killian (19) have shown that in human diabetes the diastatic activity of the blood is considerably increased. Treatment with insulin (20) reduces this blood diastase to normal figures. In the diabetic dog (21), however, the blood diastase is below normal. It is quite possible that the fluctuations which we have

observed are due to this enzyme, which we may assume to exhibit reversibility. Dialysis, or, in the case of undialyzed blood, other factors such as changes in gaseous composition, might be imagined as throwing the reaction in one direction or the other.

The increase in blood sugar at room temperature has been noted by others, although not described specifically. Several biochemists have mentioned to the writers that they have had occasion to make second or third analyses of blood, which had stood in the laboratory at room temperature, to check them for clinical reasons. Expecting lower results, they were surprised to get higher ones occasionally, not explainable on the basis of experimental error. It is apparent that the chief reason why the fluctuation of blood sugar *in vitro* has not been described heretofore is that samples of blood have not been taken for analysis at short intervals of time.

Whether the rapid decreases in blood sugar are due to condensation to a disaccharide such as was noted by Levene and Meyer (22) as a result of action of tissue extracts or to a glucosidic combination<sup>6</sup> with some non-carbohydrate component of blood is only open to conjecture. It may indicate a cleavage of the glucose molecule, although this seems improbable. Whatever the mechanism, it appears to be rapid and easily reversed.

#### SUMMARY

Short dialysis of hyperglycemic blood (diabetic or artificially fortified normal blood) frequently is followed by marked fluctuations of the blood sugar curve, after the blood has been transferred from the dialysis membrane to a glass container. However, undialyzed hyperglycemic blood may show similar fluctuations. Whether dialysis increases this tendency or not is undecided. Plasma shows the same phenomenon.

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## FAT-SOLUBLE VITAMINS

### XXXVI. THE CAROTENE AND VITAMIN A CONTENT OF BUTTER\*

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As one would expect from its general dietary importance, butter has frequently been assayed biologically for its total vitamin A activity. The published results of such assays, however, vary widely, not only because of variations in butter itself, but because of variations in the method of assay, in the method of interpreting data, and in the method of expressing results. Sherman and Smith (1) for example state, "Butter appears to contain about 30 to 50 units of vitamin A per gram, ranking in richness, weight for weight, with egg yolk and fresh spinach." Fraps and Traichler (2) find similar variations and report that butter from cows on normal diets ranges from 17 to 50 Sherman units per gm. Crawford, Perry, and Zilva (3) report the minimal daily dose of summer butter necessary to maintain growth equivalent to that produced by an excess of cod liver oil at approximately 0.1 gm.

Vitamin A activity in butter fat<sup>1</sup> is due to the presence of two different chemical entities, *viz.* carotene and vitamin A. The presence of carotene was first demonstrated by Palmer and Eckles (4) who used solubility reactions, the phase test, and the position of absorption bands in the spectrum as criteria. For the presence of vitamin A itself, evidence from various sources has been available. Stephenson (5) showed that butter fat decolorized with charcoal retained its biological activity. Morton and Heilbron (6) showed the presence of an absorption band at 328  $m\mu$ , which

\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

<sup>1</sup> For the sake of convenience the term butter is frequently used for butter fat. Actually butter contains only 85 per cent of fat.



is the region of absorption for potent liver oil concentrates. Lundborg (7) showed that the  $\text{SbCl}_3$  reagent gives a stronger blue color with the unsaponifiable fraction of butter than can be accounted for on the basis of its carotene content. Moore (8) made a similar observation, and further identified the absorption bands at  $606 m\mu$  in the  $\text{SbCl}_3$  reaction product with butter concentrate as being due to vitamin A.

Since quantitative data of the relative carotene and vitamin A content of butter are not available, it seemed advisable that a systematic study of these relations be made. Biological methods of determination, while yielding results of great practical significance, are obviously of little value in determining the relative amounts of these active components. For this reason spectrographic methods were employed. Analyses were made on fourteen samples collected in successive months during 1932 and 1933.

The butter was made from the general supply of milk delivered at the University Dairy by neighboring farmers. It represented the composite production of various breeds of cows on rations representative of Wisconsin farm feeding practise. The butter was churned from sweet cream separated by centrifugal force, and then stored at  $4^\circ$ . Samples were collected on the 15th day of each month.

For the carotene determinations the butter was melted at  $55^\circ$  and decanted from brine and curd onto a cotton filter. The intensity of the absorption bands was measured on the melted butter fat at  $30^\circ$  by means of a Bausch and Lomb universal spectrophotometer. Readings were taken from two zero points and each sample was read at two depths, selected to give results of the greatest accuracy. For purposes of calculation the absorption values at the points of maximum absorption, *i.e.* at 460 and  $485 m\mu$ , were used, and compared with the absorption maxima of a standard solution of carotene in refined cottonseed oil.<sup>2</sup> We could have used an alternative method which involved the use of extinction coefficients for carotene recorded in the literature. However, the recorded values for the extinction coefficient of carotene absorption bands vary considerably. Duliere, Morton, and Drummond (9) report that the molecular extinction coefficient,  $E$ , of the  $462 m\mu$  band in chloroform ranges from 75,000 to 100,000.

<sup>2</sup> Wesson oil.

They use the following formula,  $E = 1/cd \log I/I_0$ , where  $E$  is the molecular extinction coefficient,  $c$  the concentration in mols per liter, and  $d$  the depth of the solution in *cm.* Later, Gillam, Heilbron, Morton, and Drummond (10) reported the value for  $E$  as 102,000. Pummerer and Rebmann (11), using a different formula, report  $\log K = 5.1$  as determined in cyclohexane. Our method of determination has the advantage that we avoided the use of these published values, which after all might not be directly applicable to carotene in solution in butter fat.

The following illustrates our method based on the 485  $m\mu$  spectral line. A solution of 0.002 mg. of carotene<sup>3</sup> per cc. of cottonseed oil at a depth of 1 cm. gave a value for  $\log I/I_0$  of 0.44. July butter at a depth of 0.5 cm. gave a value of 0.86 equal to 1.72 for a 1 cm. depth. Checked at 0.75 cm. it gave a value of 1.28 equal to 1.71 for 1 cm. The value 1.72 was therefore accepted as approximately correct. Solving for  $x$  in the proportion  $1.72 : 0.44 :: x : 0.002$ ,  $x$  we found was equal to 0.0078 mg. of carotene per cc. or 0.0086 mg. of carotene per gm. of butter fat.

When the 460  $m\mu$  line was used these values were duplicated.  $\log I/I_0$  for the carotene solution at 1 cm. equaled 0.53 and for July butter 1.05 at 0.5 cm. and 1.50 at 0.75 cm., which are equal respectively to 2.10 and 2.00 for 1 cm. depth. Accepting the average, *viz.* 2.05, and solving for  $x$  in the proportion  $2.05 : 0.53 :: x : 0.002$ , we found  $x$  gave a value of 0.0078 mg. of carotene per cc. or 0.0086 mg. of carotene per gm. of butter.

The accuracy of this method of determining carotene was checked by adding carotene to February butter, as an example of a butter of minimal carotene content, and to a sample of uncolored oleomargarine in such amounts as were calculated to give these samples the color intensity of June butter. As an analysis of February butter showed that it contained 0.0022 mg. of carotene per cc. and June butter 0.0062 mg. of carotene per cc., 0.0040 mg. of carotene was added to each cc. of February butter. 0.0062 mg. of carotene was added to each cc. of oleomargarine. After these additions actual determination of the total pigmentation

<sup>3</sup> The carotene used was apparently pure  $\beta$ -carotene as it showed no optical activity and melted at 181°. It was part of a preparation sent by this laboratory to the British Medical Research Council for use in the preparation of the international standard for vitamin A.

by the spectrophotometer showed that the colored February butter now contained 0.0060 mg. of carotene per cc. and the colored oleomargarine 0.0062 mg. of carotene per cc.

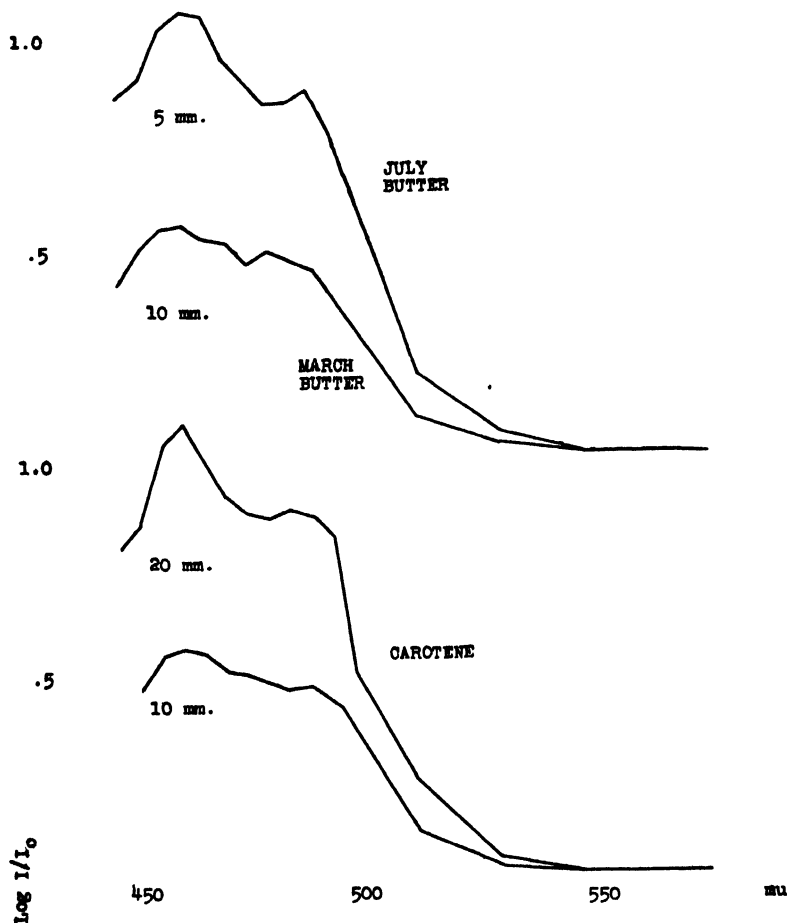


FIG 1. The spectral absorption curves in the visible part of the spectrum obtained from butter fat of high and low carotene content (July and March respectively). The observations were made with a Bausch and Lomb spectrophotometer with the butter fat at depths of 5 and 10 mm. so as to facilitate direct comparison with a solution of carotene in cottonseed oil which contained 0.002 mg in each cc. The similarity in the shape of the curves is considered significant.

That natural butter pigment consists for the most part of carotene was demonstrated as follows: 40 gm. of August butter were saponified by boiling with 12 per cent alcoholic KOH. The mixture was diluted with water and then extracted with ether. The ether was evaporated. The residue was taken up in petroleum ether. This was washed three times with an equal volume of 92 per cent methyl alcohol. It was found that 95 per cent of the pigment remained in solution in the petroleum ether. The absorption curve of the petroleum ether solution, as obtained in the spectrophotometer, was found to be identical with that of carotene (Fig. 1).

*Determination of Vitamin A*—Vitamin A was determined in the butter by photographing the intensity of the  $328\text{ m}\mu$  band of the unsaponifiable matter dissolved in methyl alcohol. Before so doing it was necessary to purify the unsaponifiable matter in order to remove so far as possible substances other than vitamin A which absorbed in the same region.

The unsaponifiable fraction was prepared as follows: 15 gm. of butter were refluxed in a stream of nitrogen for 30 minutes with 125 cc. of aldehyde-free freshly prepared 12 per cent alcoholic KOH. 125 cc. of water were added, and the mixture was cooled to  $4^{\circ}$ . 150 cc. of ether were then added to the cold solution followed by an addition of 500 cc. of cold water. The ether was drawn off and the aqueous alcoholic fraction was then extracted three successive times with additional portions of ether of 50 cc. each. The combined ether solutions were washed with water repeatedly, dried over  $\text{Na}_2\text{SO}_4$ , and freed from ether with nitrogen under reduced pressure. The residue was dissolved in 15 cc. of hot methyl alcohol and the impurities were crystallized out by cooling several hours to  $-72^{\circ}$  in a mixture of solid  $\text{CO}_2$  and acetone. The cold solutions were filtered, washed with cold, methyl alcohol, and made up to such a volume that the photographed solution showed a value at  $328\text{ m}\mu$  and a depth of 2 cm. for  $\log I/I_0$  of approximately 1.0.

Absorption spectra were photographed through a Hilger quartz spectrograph equipped with a sector photometer and a hydrogen discharge tube. Since pure vitamin A as a standard was not available, calculations of the vitamin content of the butter were made with a value for the extinction coefficient of this vitamin published by Heilbron and coworkers (12).

The following illustrates the method of calculation: A concentrate of June butter made according to the above procedure and containing the equivalent of 0.166 gm. of butter per cc. showed a value for  $\log I/I_0$  of 0.8 when photographed through a 2 cm. cell (Fig. 2). The value for the extinction coefficient of 1 gm. of butter at 328  $m\mu$  was therefore equal to  $(0.8/2) \div 0.166$ , when 2

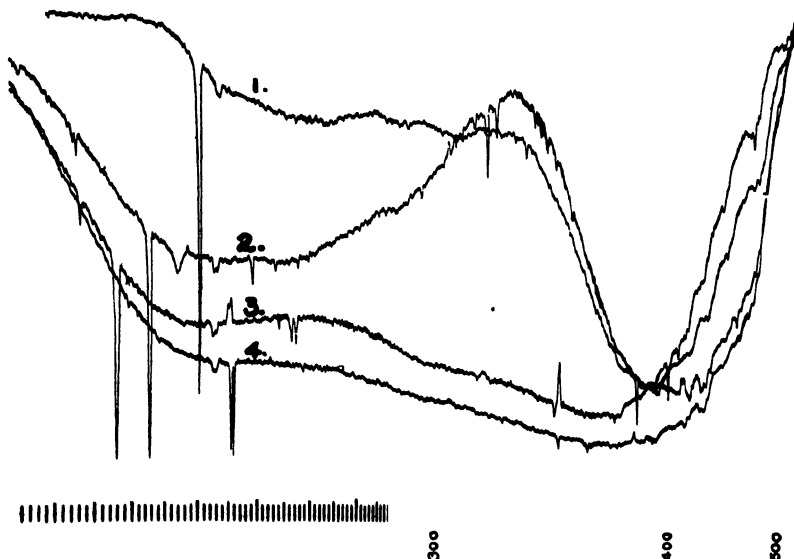


FIG. 2. Spectral absorption curves: Curve 1, a concentrate of unsaponifiable constituents from butter fat dissolved in methyl alcohol where 1 cc. = 0.166 gm. of June butter; Curve 2, a vitamin A concentrate from halibut liver oil diluted to 1:1,600,000 in methyl alcohol; Curve 3, carotene in methyl alcohol; and Curve 4, methyl alcohol. The butter concentrate shows absorption both at 450  $m\mu$  and at 325  $m\mu$ . This is accounted for on the basis of its content of both carotene and of vitamin A. Absorption at wave-lengths shorter than 300  $m\mu$  is presumably due to the presence of other substances in the butter concentrate.

The vitamin A concentrate from halibut liver oil (No. 210946) was obtained from the Abbott Laboratories, Chicago. Their biological assay indicated 1,600,000 U.S.P. units of vitamin A per gm. The antimony trichloride test showed the presence of 1,500,000 blue units per gm. measured at 3B in a Lovibond tintometer, or 3000 times as many as in standard cod liver oil. The fact that the extinction coefficient  $E$  (1 per cent concentration at a depth of 1 cm.) equaled 750 indicates that it contained approximately 60 per cent vitamin A by weight.

equals the depth of the cell in cm. and 0.166 equals the gm. of butter per cc. of the solution photographed. Therefore,  $E$  (1 gm. at a depth of 1 cm.) = 2.4 at 328  $m\mu$ .

The vitamin A values were calculated according to the Beer-Lambert law,  $E = 1/cd \log I/I_0$ , where  $E$  is the extinction coefficient,  $d$  the depth of the cell in cm., and  $c$  the concentration. If the extinction coefficient for pure vitamin A be taken as equal to 1300 (12)<sup>4</sup> and substituted in the above formula, then for June butter  $c = 0.000308$  per cent or 3.08 $\gamma$  vitamin A per cc. of solution. 0.166 gm. of butter = 3.08 $\gamma$  vitamin A. 1 gm. of June butter = 18.6 $\gamma$  vitamin A.

Several samples of each butter were analyzed by the above method and the values were averaged. That there was no loss of vitamin A due to storage was shown by the fact that preparations made from the same butter at intervals of 6 months showed the same absorption. The stability of the purified vitamin solution in methyl alcohol at 0° was likewise demonstrated.

The fact that our manipulative procedure did not result in any loss of vitamin was demonstrated as follows: A solution of April butter treated as above described showed  $\log I/I_0$  (at a depth of 2 cm.) equal to 0.83 at 328  $m\mu$  when 1 cc. contained 0.333 gm. of butter. 30 cc. of this solution, which were equivalent to 10 gm., were added to 10 gm. of April butter, and the mixture was put through the process of saponification and purification outlined above. It was made up to 60 cc. 1 cc. therefore equaled 0.166 gm. of butter plus 0.166 gm. of butter equivalent of concentrate. Any loss due to manipulation would have been reflected in a decreased absorption, since one-half of the active material had been put through two procedures, while the other half had only been put through one. However,  $\log I/I_0$  (at a depth of 2 cm.) of this solution equaled 0.83, the same as the original solution.

Some of the samples prepared showed the presence of a substance which absorbed strongly in the region of the spectrum below 300  $m\mu$ , and this substance was present in sufficient amount to absorb some of the light in the region between 320 and 330  $m\mu$  as well. The observed values for  $\log I/I_0$  at these wave-lengths

<sup>4</sup> A recent note by Carr and Jewell (13) states that the extinction coefficient of pure vitamin A is 1600. The use of this constant would lower our calculated values for vitamin A by 18 per cent.

were therefore not accurately indicative of the vitamin A content of these samples.

Substances found in butter and known to absorb in this region include carotenoid pigments, sterols, and unsaturated fatty acids. In the solutions as photographed, however, there was not enough carotene present to exert an appreciable effect upon the 328  $m\mu$  band (Fig. 1). The effect of sterols was determined by cooling saturated methyl alcohol solutions of cholesterol, ergosterol, and of irradiated ergosterol to  $-72^\circ$  in a mixture of solid  $\text{CO}_2$  and acetone as carried out with the unsaponifiable fraction of the butter fat samples. The filtered solutions were diluted with 2 volumes of methyl alcohol, bringing them to approximately the same concentration as the photographed butter solutions, if the butter solutions were saturated with sterol at  $-72^\circ$ . Analysis of these sterol solutions showed that they did not absorb at 320 to 330  $m\mu$ . The effect of fatty acids or of glycerides—possibly present in traces—was tested by resaponifying the butter concentrates and purifying them as before, since free fatty acids are known to absorb at 320  $m\mu$  (14). In no case was the absorption in the shorter ultra-violet region decreased. When 1 per cent KOH was added to the methyl alcohol solution of the purified butter concentrate, absorption in the shorter ultra-violet region increased markedly, and absorption in the 328  $m\mu$  region also increased. While alkali itself does not absorb in this region, it appeared possible that the increased absorption might be due to the effect of resins produced by the action of alkali upon aldehydes. Such aldehydes could come not only from the alcohol used in the saponification—a possibility which was minimized by using alcohol of suitable purity—but also from the oxidation and hydrolysis of unsaturated fatty acids in the butter fat. Resins produced from old alcohol showed strong absorption in the ultra-violet region.

Among methods designed to determine whether interfering agents in the butter concentrates absorb at 328  $m\mu$ , an obvious procedure would have been to remove carotene and vitamin A from the solution before analyzing the residue spectroscopically. However, the very fact that the interfering agents followed the vitamin through the purification process argued for some similarity between them and the vitamin, and procedures used to destroy carotene or vitamin A would probably, therefore, have

altered the interfering agent as well. Nevertheless, such attempts to remove vitamin A were made. Methyl alcohol solutions of vitamin A concentrates prepared from butter and halibut liver oil were transferred to ether and excessively exposed to light. They were first irradiated in air for 4 hours under a Sunshine carbon arc followed by 4 hours exposure to the rays of a cold quartz lamp. During these exposures a Pyrex filter which removed radiations below  $325\text{ m}\mu$  was used to prevent the destruction of

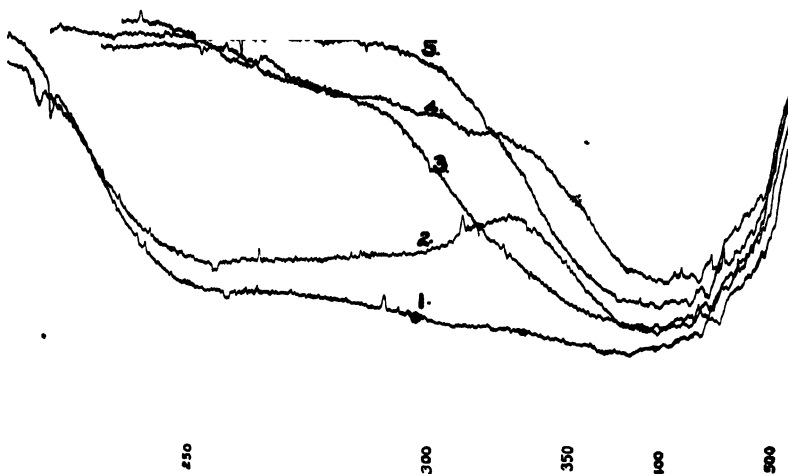


FIG. 3. Spectral absorption curves: Curve 1, methyl alcohol; Curve 2, a vitamin A concentrate (the same as in Fig. 2) from halibut oil diluted 1:3,200,000 with methyl alcohol; Curve 3, the same solution oxidized; Curve 4, a concentrate of unsaponifiable constituents from butter fat (1 cc. = 0.2 gm. of August butter); Curve 5, the same solution oxidized. Oxidation of both the butter and the halibut concentrates resulted in decreased absorption at  $325\text{ m}\mu$  and a very marked increase in absorption below  $300\text{ m}\mu$ .

those agents which absorb in this region. The ethereal solutions were then transferred to Pyrex tubes and exposed to light from a window of northern exposure for 3 days. Spectroscopic examination of these solutions showed very little absorption in either the  $325\text{ m}\mu$  region or in the shorter ultra-violet region.

An attempt was also made to destroy selectively vitamin A and carotene in butter concentrates by oxidation. The concentrates were transferred to toluene, and air was bubbled through them



for 3 days at room temperature. After evaporation of the solvent, the residues were dissolved in methyl alcohol. Spectroscopic analysis showed that oxidation of a vitamin A concentrate from halibut liver oil resulted in a disappearance of the 328  $m\mu$  band and the appearance of general absorption in the shorter ultra-violet region. The new band showed a value for  $\log I/I_0$  at 280  $m\mu$  many times larger than that of  $\log I/I_0$  at 328  $m\mu$  for an unoxidized solution of the same concentration. While the oxidized halibut liver oil concentrate showed some absorption at 328  $m\mu$  this absorption was much less than that observed at shorter wavelengths (Fig. 3). Oxidation of the butter concentrates resulted in a marked increase in absorption in the shorter ultra-violet region, and such oxidized concentrates also absorbed at 325  $m\mu$ . On dilution, however, this absorption decreased. It therefore appears that the interfering agents in the original butter concentrates are oxidation products of carotenoid substances and that these substances do not appreciably absorb light at 325  $m\mu$ , when their concentration as measured by the absorption at 280  $m\mu$  is relatively low.

Since the absorption curves of the butter concentrates all showed the presence of substances in addition to carotene and vitamin A, as evinced by absorption below 300  $m\mu$ , a minimum effect of these unknown substances upon the 328  $m\mu$  band was insured by arbitrarily discarding all analyses in which the absorption at 280  $m\mu$  exceeded that at 328  $m\mu$ .

Parenthetically it may be stated that in connection with another research in progress at this laboratory, samples of butter made from irradiated milk were analyzed for their content of carotene and of vitamin A. No loss of either component was observed although the milk had been exposed to ultra-violet rays sufficiently to have imparted to it a potency of 50 Steenbock units of vitamin D per quart. The acidity of the cream at churning time was likewise without effect. Butter made from neutral cream contained the same amount of carotene and vitamin A as that made from cream with an acidity of 0.42 per cent lactic acid.

The results of our carotene and vitamin A determinations are shown collectively in Table I. The carotene values ranged from a minimum of 2.0 $\gamma$  per gm. of butter fat in April to a maximum of 8.6 $\gamma$  per gm. in July. Vitamin A values ranged from 9 $\gamma$  in April

to 20 $\gamma$  in August. A general parallelism was found between the carotene and the vitamin A content of the samples. However, the July butter presented an anomaly, being lower in vitamin A content than either the June or August samples. We are unable to explain this. The error did not arise through storage of the butter since the irradiated July butter was also lower in vitamin A content than irradiated June and August butter. It, however, is

TABLE I  
*Seasonal Variation in Carotene and in Vitamin A Content of Butter*

Date	Carotene per gm butter fat	$E_{1\%}^{1\text{cm}}$ gm butter at 325 $m\mu$	Vitamin A per gm butter fat*
	$\gamma$		$\gamma$
Feb, 1932	2 2	1.4	11
Mar, 1932	2 2	1 1	9
Apr, 1932	2 0	1 1	9
May, 1932	2 4	1 7	14
June, 1932	7 1	2 4	19
July, 1932	8 6	1 9	15
Aug, 1932	5 8	2 5	20
Sept, 1932	5 2	2 4	19
Oct, 1932	4 5	2 1	17
Nov., 1932	4 0	2 0	16
Dec, 1932	3 0	1 9	15
Jan, 1933	2 4	1 5	12
Feb, 1933	2 2	1 5	12
Mar, 1933	2 0	1 3	10

\* The values for vitamin A are calculated on the assumption that the absorption at 325  $m\mu$  is due entirely to vitamin A. Bills has recently shown that when this assumption is applied to liver oils, the maximum variation between biological and spectrographic results is 20 per cent; and the average is less than 10 per cent (15).

possible that the cream from which this butter was made had been partly collected from another source. We had no means of checking this up.

In order to calculate the relative importance of carotene and vitamin A in the extent to which they contribute to the vitamin A activity of butter, we may tentatively accept Sherman's figure of 50 Sherman rat units per gm. as the biological value of our summer butter. Preliminary observations indicate that 1 Sherman unit

is equal approximately to 1.3 international units—1 international unit being the activity of 1 $\gamma$  of standard carotene. Sherman's value for summer butter then becomes 65 international units of which, according to our determinations, no more than 8 units are due to carotene.

Another method of calculation involves the acceptance of vitamin A values. Euler and Karrer (16) and Drummond (12) report that for a rat the daily minimum physiological dose of pure vitamin A which is capable of inducing growth lies between 0.1 and 0.5 $\gamma$ . With the average value 0.3 taken as a basis for our calculations 20 $\gamma$  of vitamin A in summer butter therefore equal 66 biological units or 85 international units of vitamin A. 8 $\gamma$  of carotene equal 8 international units of carotene in the same butter.

The two methods of calculation are not in exact agreement, the reason for which, in view of the assumptions made, can be easily understood. Apparently the main difficulty in securing better agreement at present lies in the large variation in the response of different animals to a uniform dosage of active material. It, however, appears that less than 15 per cent of the biological activity in butter is due to carotene. From a practical point of view the important difference in the biological potency of summer and winter butter is due to its content of vitamin A as such. The use of carotene as an artificial butter color would not materially increase the biological potency of the winter butter unless enough pigment were added to color the butter a deep red.

The variations in the carotene and the vitamin A content of butter are of some physiological interest since the carotene content varies 400 per cent, whereas the vitamin A content varies only 200 per cent. This is not a surprising result when the diet contains an excess of carotene, for carotene is obtained directly from the diet and would be expected to vary more widely than vitamin A, as the latter is a product of animal metabolism. The well known capacity of the liver to store excess vitamin A could easily account for the greater constancy of this component in the butter fat. Copeland and Fraps (17) and Moore (8) have calculated the relation between the vitamin A activity of summer butter and the vitamin A activity of a cow's diet when on pasture. Both workers find an enormous excess of dietary vitamin A. It appears that the problem of increasing the vitamin A content of summer butter

by means of dietary adjustment is not promising because the animal is already well supplied with carotene in proportion to its ability to convert it into vitamin A. On the other hand, the problem of adjusting the winter diet of the cow so as to maintain the summer level of vitamin A in milk and butter fat is one worthy of consideration.

#### SUMMARY

Spectrographic methods of analysis, which detect small variations in the occurrence of substances showing selective spectral absorption revealed that the vitamin A activity of butter can be accounted for on the basis of its content of carotene plus vitamin A. In the sum total of biological activity carotene is not of major importance, since it accounted for only 15 per cent of the total. Quantitatively the amount of carotene ranged from 2.0 $\gamma$  per gm. of April butter to 8.6 $\gamma$  per gm. of July butter. Vitamin A values ranged from 9 $\gamma$  per gm. of April butter to 20 $\gamma$  per gm. of June butter. These variations were seasonal and regular. No loss of either active component was observed as the result of storage for 6 months at 0°, or in consequence of ultra-violet irradiation of the milk from which the butter was made. Carotene additions to winter butter in an attempt to increase the vitamin A activity up to that of summer butter do not appear to be practical unless the public should be found to be willing to accept a much more highly colored product than it does at present.

We are indebted to Professor H. C. Jackson and to Mr. K. G. Weckel of our Dairy Department for the preparation of the butter samples. To them we wish to express our appreciation.

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## FAT-SOLUBLE VITAMINS

### XXXVII. THE STABILITY OF CAROTENE SOLUTIONS\*

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The prominent position of carotene in present day research as a result of the discovery that it can function as the precursor of vitamin A and its resultant adoption as the international standard of reference for vitamin A activity (1) make it imperative that data on its stability be accumulated. In the crystalline state, under special conditions, carotene has been reported remarkably stable. Thus one of Arnaud's preparations, sealed in a tube of hydrogen, was examined after 40 years and found to possess biological activity (2). Unfortunately, biological assays must be conducted with carotene solutions, and evidence is abundant that carotene in solution may be quite unstable. Drummond, for example, using ethyl oleate as a solvent reported carotene to be without biological activity (3). This finding was later explained when it was shown that carotene rapidly deteriorates in that solvent (4, 5). In ethyl laurate a decolorization was also found to take place (4). Gross observations made in this laboratory have indicated that carotene is unstable in olive oil, coconut oil, and mineral oil under the conditions imposed by their use in biological assays. On the other hand, carotene has been reported stable in olive oil and paraffin oil at 0° (4). A recent note by McDonald (6) states that carotene is unstable in ethyl butyrate, ethyl laurate, ethyl palmitate, and in peanut oils, but that in cottonseed oil, maize oil, and cod liver oil only 8 per cent of the pigment is destroyed in 4 weeks. The wide use of cottonseed, arachis, and corn oil as carotene solvents in biological assays might be taken as a

\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

suggestion that at least no gross destruction of the pigment occurs in these solvents. Nevertheless, the belief has arisen that no solvent exists which is absolutely safe for use in the preparation of a stable carotene solution.

Mattill believes that the stability of carotene in solution is dependent upon the nature and amount of antioxidant which may be present (7). He has shown that carotene is stable in ethyl laurate in the presence of hydroquinone, and furthermore that the rate of destruction of carotene in ethyl oleate is markedly decreased by the addition of this antioxidant. Hydroquinone was also found to stabilize carotene dissolved in chloroform, xylene, and paraffin oil. The relatively greater stability of carotene in most vegetable fats as compared with animal fats has been attributed to a difference in the amounts of antioxidant present (8). In contrast with protective catalysts—such as antioxidants—there are also known substances which accelerate the destruction of carotene. Iron salts (9) and unsaturated fatty acids (10–12) are catalysts of this type.

For several years we have used a purified cottonseed oil as a solvent for carotene and as a diluent for cod liver oil in connection with biological assays with considerable success. In view of this experience we chose this solvent in studies of the effect of concentration, temperature, light, air, rancidity, and of certain catalysts on the stability of carotene. However, we also used other oils, the ethyl esters of lauric and sebacic acids, and the ordinary organic solvents for comparison. An obvious limitation in the selection of a solvent for feeding experiments is, of course, the necessity of its being relatively physiologically inactive.

For the present experiments the carotene solutions were prepared as follows: Pure carotene, m.p. =  $181^{\circ}$ ,<sup>1</sup> was dissolved in ether, the oil in question was added to the ether, and the ether was evaporated under reduced pressure in a stream of nitrogen. The addition of hydroquinone was effected by preparing a standard 2 per cent solution of hydroquinone in ethyl alcohol and adding an aliquot to the ether solution of carotene and oil. The oil solutions were stored in small test-tubes, 8 mm. in diameter and

<sup>1</sup> The carotene used was part of the same preparation sent by this laboratory to the British Medical Research Council for use in the preparation of the international standard for vitamin A.

75 mm. long, each tube being filled approximately three-fourths full. They were tightly stoppered.

The stabilizing action of various oils upon carotene was studied by storing them in the refrigerator at 4°. Two samples of each type of oil solution were used, one containing hydroquinone equivalent to 10 per cent of the weight of the carotene, the other containing no hydroquinone. The tests were run with the following oils: cottonseed oil, olive oil, corn oil, wheat germ oil, coconut oil, and sesame oil. In addition ethyl laurate and diethyl sebacate were compared with the oils. The oils were of a commercial grade and were not subjected to purification. They showed the following acid numbers: cottonseed oil 0.2, corn oil 0.2, sesame oil 0.14, olive oil 2.0, coconut oil 19.0, and wheat germ oil 40.0. The esters were made from commercial samples of the corresponding acids.

To study the effect of various known factors on the stability of carotene, solutions of carotene in cottonseed oil were stored in the dark respectively at 4°, at room temperature (approximately 20–25°), at 37°, and exposed to the diffuse light of the laboratory at room temperature. In order to simulate the treatment given to carotene solutions when used in biological assays, a group of preparations was stored at 4° but taken into the laboratory and uncorked for 5 minutes each day. Four samples of solution were stored under each of the above conditions. Two contained hydroquinone equivalent to 4 per cent of the carotene, and two contained no hydroquinone. In one of each of these pairs the air space was replaced by nitrogen. Since our routine method of dissolving carotene in oil consisted of first adding both components to ether, and subsequently removing the ether, it seemed desirable to determine what effect traces of this solvent would exert upon the stability of the carotene. 0.5 cc. of ether was therefore added to 3 cc. of the oil solutions.

Carotene was also dissolved in a mixture of equal parts of cottonseed oil and of wheat germ oil, since wheat germ oil is supposedly rich in antioxidants (8, 13). To determine the effect of changes within the oil on the stability of carotene, a very rancid sample of cottonseed oil was used as a solvent and stored as before.

The effect of concentration upon stability was studied with purified cottonseed oil as an example of a stabilizing solvent, and



ethyl laurate as an example of a solvent in which destruction of carotene is comparatively rapid. The original ethyl laurate solutions contained 0.375, 0.128, 0.026, and 0.006 mg. of carotene per cc. The solutions in cottonseed oil contained 0.467, 0.136, 0.033, and 0.010 mg. of carotene per cc. The solutions were stored at room temperature in tightly corked, half filled test-tubes, and were examined periodically as described below.

The stability of carotene in the common organic solvents and in certain esters was studied in a manner similar to that employed in studying stability in the edible oils. Carotene was dissolved in the carefully purified solvent, and stored in the refrigerator at 4°. For the 1st month aliquots were analyzed weekly; thereafter, monthly. Two samples of solutions in each solvent were stored; one contained only carotene, the other, carotene with hydroquinone added in amounts equal to 10 per cent of the original carotene content.

The carotene was determined by means of a Bausch and Lomb spectrophotometer. Aliquots of the carotene solution were dissolved in chloroform and the intensity of the absorption bands at 485 and 460  $m\mu$  were measured directly. Readings were taken in the direct and in the reverse position and at two depths for each band. Thus, each carotene determination represents an average of eight readings. However, in spite of this there were observed variations in the results from week to week which were probably caused by variations in the method of sampling. The method of calculation has been described previously (14).

Of the various oils which were used as solvents for carotene, a refined cottonseed oil was found to be the most satisfactory. When stored at 4° in the dark for as long a period as 5 months, the amount of carotene which disappeared was as follows: refined cottonseed oil 8 per cent, sesame oil 15 per cent, coconut oil 25 per cent, olive oil 38 per cent, corn oil 45 per cent, and crude wheat germ oil 62 per cent. These results are shown collectively in Table I which also presents the data obtained with the same oils after hydroquinone additions. When hydroquinone was added in amounts equal to 10 per cent of the weight of the carotene, no effect upon carotene destruction was observed. However, in the case of two esters, namely ethyl laurate and ethyl sebacate, which were included in this series, hydroquinone increased the stability

of carotene markedly. For example, in ethyl laurate without hydroquinone 72 per cent of the pigment disappeared in 2 months and 99 per cent in 5 months. With hydroquinone the loss after 5 months was reduced to 12 per cent. In the case of ethyl sebacate 63 per cent of the carotene disappeared in 2 months and 97

TABLE I  
*Stability of Carotene in Various Oils at 4°*

Oil	Original concentration of carotene	Carotene loss in per cent						
		1 wk	2 wks	3 wks	4 wks	2 mos	3 mos	5 mos
	<i>mg per cc</i>							
Cottonseed*...	0 260			0	0	10	7	9
“ + HQ†	0 200	0	0	1	4	12	15	16
Sesame‡	0 111		0				15	
“ room temperature	0 111		24				81	
Olive§.	0 200	0	0	8		28		38
“ + HQ	0 200	0	0	0		20		39
Corn	0 200	0	0	0	5	20		41
“ + HQ	0 200	0	0	1	14	23		52
Wheat germ¶	0 220	0	11	16	39	42	55	60
“ “ + HQ	0 220	0	2	20	40	32	58	64
Coconut	0 190	3	5	0	7	24	28	26
“ + HQ	0 195	0	0	0	5	10	18	25
Ethyl laurate	0 190	6		6	31	72	88	99
“ “ + HQ	0 190	0	0	0	6	11	11	12
“ sebacate	0 195	10	11	10	39	63	86	97
“ “ + HQ.	0 200	0	5	0		11	8	8

\* Wesson oil brand.

† Hydroquinone

‡ We are indebted to the Globe Grain and Milling Company, Los Angeles, for this sample

§ Pompeian oil brand

|| Mazola brand.

¶ We are indebted to E R Squibb and Sons for this sample.

per cent in 5 months without hydroquinone. With hydroquinone only 8 per cent was destroyed.

In Table II there are presented data on the stability of carotene in refined cottonseed oil under various conditions. After 5 months storage at 4°, the temperature of our refrigerator, only 8 per cent of the pigment was found destroyed. In the same period of time

at room temperature there occurred a 30 per cent loss when the samples were stored in the dark, a 37 per cent loss when the samples were exposed to laboratory light, and a 48 per cent loss when they

TABLE II  
*Stability of Carotene in Refined Cottonseed Oil under Varying Conditions*

Storage conditions	Original concentration of carotene	Carotene loss in per cent						
		1 wk.	2 wks.	3 wks.	4 wks.	2 mos.	3 mos.	5 mos.
	mg. per cc.							
Dark, 4°, N <sub>2</sub> .....	0.263	3	6	0	6	5	10	
Same + HQ.....	0.200		0	0	0	7	5	10
Dark, 4°, air.....	0.260	1		0	0	10	7	9
Same + HQ.....	0.200	0	0	1	4	12	15	16
Samples opened daily, dark, 4°, N <sub>2</sub> .....	0.312	0		13	26	26	33	38
Same + HQ.....	0.245	0	17	21	26	28	30	40
Samples opened daily, dark, 4°, air.....	0.312	0	4	6	21	24	28	49
Same + HQ.....	0.270	0		24	30	34	39	66
Dark, room temperature, N <sub>2</sub> ....	0.278	0	7	6	17	21	19	30
Same + HQ.....	0.200	2	3		16	20	35	63
Dark, room temperature, air.....	0.265		0	0	10	8	15	28
Same + HQ.....	0.200		0	0	10	12	22	29
Light, room temperature, N <sub>2</sub> ....	0.260	2		6	16	17	22	36
Same + HQ.....	0.200	0		0	4	22	20	37
Light, room temperature, air.....	0.260		0	0	8		7	46
Same + HQ.....	0.200	0		0	5	14	15	29
Dark, 37°, N <sub>2</sub> .....	0.250	6	6	14	21	29	51	60
Same + HQ.....	0.200	10	15	24	32	53	84	99
Dark, 37°, air.....	0.250	12		25		61	99	99
Same + HQ.....	0.200	15	28	21	26	51	72	99
Dark, 4°, rancid oil.....	0.205	0	0	0	0	0	0	22
Same + HQ.....	0.210	0	0	0	0	0	0	10
Dark, 4°, ether.....	0.168	0	0	0	0	3	0	0
Same + HQ.....	0.180	0	0	0	0	16	16	15
Dark, 4°, wheat germ oil.....	0.204		0	0	6	16	6	16
Same + HQ.....	0.200		0	0	3	15	5	15

were exposed to laboratory air and light for 5 minutes daily but kept for the rest of the time at 4° in the dark. It will be noted from these results that not only an increase in temperature but also an exposure to light and to air accelerated the deterioration.

Under the various conditions which were imposed, stability was not increased by the addition of hydroquinone equivalent to 4 per cent of the weight of the carotene or by passing nitrogen through the oil before stoppering the container. It is possible that the nitrogen did not exercise a demonstrable effect because the "air space" in the storage tubes was small, but we cannot come to any conclusion in regard to this because experiments in which this factor was varied were not carried out. Furthermore wheat germ oil which is credited with containing antioxidants had but little stabilizing effect. In a mixture of equal parts of cotton-

TABLE III  
*Effect of Concentration upon Stability of Carotene Solutions at Room Temperature*

Solvent	Original concentration	Carotene loss in per cent				
		1 wk	3 wks.	2 mos	3 mos	5 mos
Ethyl laurate	<i>mg per cc.</i>					
	0 37	55	74	95		
	0 13	16	76	98		
	0 02	31	59	95		
Cottonseed oil	0 006	9	79	99		
	0 46	22	31	31		42
	0 13		9	18	30	57
	0 03	0		0	6	34
	0.01	0		0	21	32
Wesson oil + excess undissolved hydroquinone	0 20			26		27*

\* 4 instead of 5 months.

seed oil and of wheat germ oil 14 per cent of the carotene originally present was found to be destroyed in 5 months. Possibly this was influenced by the high acid number of the wheat germ oil. Curiously enough, we also observed but little deterioration in consequence of using rancid cottonseed oil in place of the neutral solvent. The addition of ether which was added purposely in order to determine any effect which ether might have as a contaminant also was found to have no effect on the stability.

From the results presented in Table III, in which carotene was tested for its stability at various concentrations with cottonseed

oil and ethyl laurate as the solvents, it is evident that decomposition in ethyl laurate proceeded apace irrespective of the amounts of carotene present. In refined cottonseed oil the carotene, as

TABLE IV  
*Stability of Carotene in Common Organic Solvents at 4°*

Solvent	Original concentration of carotene	Carotene loss in per cent						
		1 wk	2 wks	3 wks	4 wks	2 mos	3 mos	5 mos
	mg per cc							
Ether	0 160	0	20	37	45	92	96	100
“ + HQ	0 200	0	16	17	30	66	78	93
Chloroform	0 160	8	19	35		36	62	79
“ + HQ	0 150	13	25	30		37	43	68
Petroleum ether	0 105	14	15			20	28	46
“ “ + HQ	0 120	35	34	35		32	29	35
Benzene	0 093	0		1		12	15	23
“ + HQ	0 085	2	7	14				10
Cyclohexane	0 095	0	11	14		61	91	99
“ + HQ	0 095	2	6	6		5	16	18
Acetone	0 081	34	48	60		87	93	98
“ + HQ	0 080		0	11		39	75	63
Carbon disulfide	0 260	7	14	21		31	59	96
“ “ + HQ	0 246	0		0		0	0	0
Toluene	0 111	25	28	28		27	39	78
“ + HQ	0 090	0	0	4		16	16	18
Pyridine	0 241	11	20	29		52	79	89
“ + HQ	0 266	0	0			2	10	13
Methyl alcohol*	0 005		1				56	
Ethyl “ *	0 011		56				50	
n-Propyl “ *	0 031		32				55	
n-Butyl “ *	0 032		26				61	
95 per cent ethyl alcohol*	0 005		0				27	
Alcohol-carbon disulfide mother liquors*	0 036		72				87	

\* These were stored at room temperature because at 4° the carotene precipitated out.

was to be expected, was not destroyed so rapidly, but destruction nevertheless took place probably because these experiments were run at room temperatures. However, the amounts destroyed were not markedly different with varying concentration, but there

appeared to be less destruction at the lower than at the higher concentrations of carotene. As a matter of fact, there occurred considerable differences in the series which bore no relation to concentration. It is possible that these were caused by variations in exposure to air and variations in mixing the contents at the time that the periodic examinations were made.

TABLE V

*Stability of Carotene in Certain Esters*

All solvents were maintained at 4° with the exception of that of the experiment on ethyl acetate exposed to air, which was kept at room temperature

Solvent	Original concentration	Carotene loss in per cent					
		1 wk	2 wks	3 wks	4 wks	2 mos	3 mos
	<i>mg per cc</i>						
Ethyl laurate	0 375	0	20			91	98
" " opened daily	0 375		99				
" stearate	0 366	42	75	95		95	
" " opened daily	0 366		99				
" levulinate	0 114	63	98				
" malonate	0 100				91		
" " opened daily	0 100	77	81				
" succinate	0 158	0					36
" " opened daily	0 158	0	0	1	19		
" acetate	0 200			5			24
" " air	0 200			99			
Crude glyceryl acetate	0 102				20		90
" " " opened daily	0 102		32		31		
Glyceryl diacetate	0 095	27	46				
" " opened daily	0 095		65				65
" triacetate	0 092		89				90
" " opened daily.	0 092	78	94				

Table IV shows the results that were obtained with twenty-four different organic solvents which are frequently used to dissolve lipids. These carotene solutions were kept at 4°. In ethyl and methyl alcohols and in 95 per cent ethyl alcohol the carotene was found to crystallize out. Measurements in these cases would consequently have been essentially determinations of the stability

of carotene covered with these solvents and therefore no emphasis was placed on these. However, gross observations indicated that the crystals were approximately stable for 5 months. In benzene the carotene loss was 23 per cent and in petroleum ether 46 per cent in 5 months. In ether, chloroform, cyclohexane, acetone, carbon disulfide, toluene, and pyridine more than 75 per cent of the pigment was destroyed in 5 months. However, in all of these solvents the addition of hydroquinone had a marked effect. After its addition less than 20 per cent of the original carotene was found to be destroyed in carbon disulfide, benzene, pyridine, toluene, and cyclohexane. With petroleum ether 35 per cent of the carotene was destroyed and with acetone, chloroform, and ether 60 per cent or more. In the alcohols, namely ethyl, propyl, and butyl, 50 to 60 per cent loss occurred in 3 months at room temperature.

In the presence of various esters carotene reacted in a variable manner (Table V). It was found to be markedly unstable in the ethyl esters of stearic, lauric, levulinic, and malonic acids, and in the di- and triacetates of glycerol. Most of the pigment had disappeared within a month. However, in ethyl acetate and in ethyl succinate carotene was comparatively stable when kept at 0°. Even when exposed periodically to room temperature and to light, which is unavoidable when used for feeding purposes, ethyl succinate retained its carotene content for 3 weeks without loss.

#### SUMMARY AND DISCUSSION

As a stabilizing solvent for carotene, refined cottonseed oil was found to be outstanding among the common edible oils. At 4° carotene was found to be stable in sesame oil as well, but at room temperature destruction of the pigment proceeded much more rapidly in sesame oil than in the refined cottonseed oil. All other oils were markedly inferior. Ethyl laurate and diethyl sebacate, when fortified with hydroquinone, were found equal to cottonseed oil, although unfortified these solvents were very unsatisfactory.

On the basis of available data no broad generalization on the mechanism of attaining carotene stability is warranted since decolorization of carotene may proceed in at least two ways; *e.g.*, by simple oxidation, or by conversion to achroocartene (7) which

is claimed not to be an oxidation product. Our experiments were not designed to determine the character of the mechanism involved. Nevertheless, a few comments may be hazarded. In cottonseed oil carotene losses were not reduced by the addition of small amounts of hydroquinone, by replacing the air with nitrogen, or by the addition of an antioxidant-containing material like wheat germ oil. This would suggest that the cottonseed oil already contained an adequate amount of antioxidant. Such an assumption is further suggested by the fact that supposedly destructive substances like ether and rancid oil were without effect. Although the acidity of the oil may influence stability, it is not necessarily the dominant factor. Thus, carotene losses were greater in corn oil with an acid number of 0.2, than in coconut oil with an acid number of 19.0. On the other hand carotene losses were highest in wheat germ oil with an acid number of 40. Concentration, likewise, may or may not affect stability. In ethyl laurate no relationship was found; in cottonseed oil, on the contrary, carotene was more stable at the lower than at the higher concentrations. If we assume the presence of protective substances in the oil, the ratio of protective substance to carotene will vary inversely as the concentration of the carotene, and hence, more protection is available at the lower concentrations.

Exposure to light, or air, or higher temperature definitely increased the rate of carotene destruction in cottonseed oil. However, this increase in the rate of destruction varies with different oils, and presumably also with the sample (15). Thus, carotene is fairly stable in olive or coconut oil at 4°, and highly unstable under the conditions unavoidable in the use of a preparation for feeding purposes, when the losses may amount to 95 per cent within a month. In cottonseed oil, on the contrary, the loss during the 1st month is only 26 per cent under these conditions. Since under feeding conditions there may be loss of carotene during the 2nd week, it is recommended that the main supply of solution be kept at 0° in the dark, and that the aliquots necessary for a week's feeding be removed periodically.

The stability of carotene in organic solvents bears no obvious relationship to the structure of the solvent molecule. Thus carotene is relatively stable in ethyl and methyl alcohols, a stability similar to that reported by Dann (16) for vitamin A in these



liquids. In the other solvents it is relatively unstable. In the esters there is also a lack of correlation. Thus carotene is stable in ethyl acetate and ethyl succinate, but markedly unstable in the esters of such widely varying acids as malonic, stearic, and levulinic.

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## THE HEMICELLULOSES OF MESQUITE WOOD\*

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Recent work has repeatedly demonstrated a striking similarity in the composition of pectins, plant gums, and hemicelluloses and has naturally led to suppositions concerning the generic relationship which may exist between these substances. The experimental basis for these suppositions is, for the most part, no more fundamental than this similarity in structure. It is true, O'Dwyer (1) showed that in unligified tissue the ratio of pectin to hemicellulose is large, while in lignified tissue it is small, the pectin having almost completely disappeared. This led her to suggest the transition of pectin into hemicellulose. Candlin and Schryver (2) heated pectin with 0.5 per cent sodium hydroxide and obtained products which resembled hemicellulose and which hydrolyzed to give sugars and uronic acids. Norman (3) brought about a similar change by mild oxidation of pectin. He suggested that the plant gums may well have the same origin as the hemicelluloses which they so closely resemble.

The similarity in the constitution of the hemicelluloses and plant gums is generally more marked than that existing between pectin and either class of compounds. While the nucleus of the gums and hemicelluloses is more frequently an aldobionic acid, the nucleus of the pectin molecule contains the tetrauronic acid group. Pectin has, on the whole, a higher content of uronic acid.

Because of the scarcity of accurate data concerning hemicelluloses, their study presents an interesting problem in itself. But since the constitution of mesquite gum has been so completely established (4), the present investigation was undertaken to deter-

\* Most of the material reported in this contribution was taken from a dissertation presented by Wilbur Y. Gary in partial fulfilment of the requirements for the degree of Master of Science at the University of Arizona.

mine what relation, if any, exists between the hemicellulose of mesquite wood and the gum which the wood exudes. Such a study might throw light upon the origin of the gum.

#### EXPERIMENTAL

*Extraction and Preliminary Fractionation of Hemicellulose*—The wood used in this investigation was cut a year in advance. The bark and worm-eaten exterior were removed from three short logs 4 to 8 inches in diameter and the remainder was cut into sawdust.

3 kilos of sawdust were rendered fat-free by extraction with ether. The pectin was removed by extracting twice with 0.5 per cent ammonium oxalate in the boiling water bath for periods of 2 hours. Protein, some lignin, and much coloring matter were removed by extraction with 1 per cent ammonium hydroxide for 24 hours. The hemicellulose was removed by extracting the wood twice, each time for 48 hours with 4 liters of 5 per cent sodium hydroxide. The combined extracts were neutralized and then made definitely acid with hydrochloric acid. The acid caused the formation of a dark brown precipitate which was separated from the liquid and called Hemicellulose A in conformity with the nomenclature suggested by Norris and Preece (5). The addition of a half volume of acetone to the filtrate did not precipitate a Fraction B corresponding to their Hemicellulose B but the addition of 1 volume of acetone caused the formation of Hemicellulose C.

*Purification of Hemicellulose A*—A great deal of lignin and coloring matter were removed from the hemicellulose by washing it thoroughly with acetone. The hemicellulose was then dissolved in 2.5 per cent sodium hydroxide and precipitated by adding ethanol until the concentration became 55 per cent. After precipitating the hemicellulose with ethanol from alkaline solution several times, a colorless filtrate resulted. It was then dissolved in 2.5 per cent sodium hydroxide, acidified with acetic acid, and precipitated with ethanol at a concentration of 55 per cent. The introduction of the acid rendered soluble an additional amount of coloring matter; therefore, the hemicellulose was repeatedly precipitated from an acidified solution until a colorless filtrate indicated the futility of continuing the process.

*Fractionation of Hemicellulose A*—The hemicellulose was dissolved in 2 liters of 2.5 per cent sodium hydroxide. Ethanol,

added until its concentration became 35 per cent, caused the formation of a heavy precipitate. This was removed and subjected to a second precipitation under the same conditions in order to free it from any occluded material. This precipitate was called Fraction A<sub>1</sub>. The addition of ethanol to the filtrate from Fraction A<sub>1</sub> gave no further precipitate until its concentration was above 50 per cent. Precipitation was complete at a concentration of 55 per cent of ethanol. The precipitate was filtered off and called Fraction A<sub>2</sub>. In this way the hemicellulose was divided into two fractions differing distinctly in their solubilities.

In order to remove the inorganic ions more completely, both fractions were peptized in separate quantities of warm water, rendered 5 per cent acid with acetic acid, and precipitated with ethanol at a concentration of 55 per cent. This was repeated until a small sample of the filtrate evaporated to dryness showed practically no inorganic residue. The hemicelluloses were freed from water in the usual way by successively suspending them in alcohol of increasing concentrations. They were finally washed with ether and dried in a desiccator.

*Purification and Fractionation of Hemicellulose C*—Hemicellulose C was purified by the method described above. Most of the impurities extracted from the wood by the alkali were precipitated with Fraction A, consequently fewer precipitations were required to purify Hemicellulose C. Although there was not the same sharp indication that the hemicellulose was being divided into two separate and distinct substances as in the case of Hemicellulose A, it was arbitrarily separated into Fractions C<sub>1</sub> and C<sub>2</sub> by acidifying the solution and adding the same concentrations of ethanol as were used in fractionating Hemicellulose A.

The yields of the various fractions of hemicellulose obtained were as follows: Fraction A<sub>1</sub>, 42.55 gm. or 1.41 per cent of the air-dried wood; Fraction A<sub>2</sub>, 3.82 gm. or 0.13 per cent; Fraction C<sub>1</sub>, 31.11 gm. or 1.04 per cent; Fraction C<sub>2</sub>, 6.12 gm. or 0.20 per cent.

The dry hemicelluloses varied from a light tan color in the case of Fraction A<sub>1</sub> to almost pure white in the case of Fraction C<sub>2</sub>. Their solubility in water increased in the order of Fractions A<sub>1</sub>, A<sub>2</sub>, C<sub>1</sub>, C<sub>2</sub>. Fraction A<sub>1</sub> was very slightly soluble while Fraction C<sub>2</sub> gave a clear brownish solution in warm water. A test for starch showed the slightest trace in Fraction A<sub>1</sub> but was entirely negative in the other fractions.

*Analysis and Composition of Hemicelluloses*—All four fractions of the hemicellulose gave carbon dioxide when boiled with 12 per cent hydrochloric acid. After they had been hydrolyzed in hot normal sulfuric acid for 2 hours they gave a faint but unmistakable naphthoresorcinol test. Consequently all fractions were polyuronides.

When the hemicelluloses were hydrolyzed, an insoluble residue was left in every case. This was reported as body X according to the nomenclature of Anderson and Kinsman (6).

TABLE I  
*Analyses of Hemicelluloses*

Hemicellulose		Uronic anhy- dride	Xylan	CH <sub>2</sub> O	Body X	Total accounted for*	Mol wt based on uronic anhydride
		per cent	per cent	per cent	per cent	per cent	
Fraction A <sub>1</sub>	Found	10 16	84 49	1 96	6 57	102 18	1732
" "	Theory	10 02	82 64	1 77	6 57	101 00	1757
" A <sub>2</sub>	Found	17 44	79 06		5 16	101 66	1009
" "	Theory	17 00	76 52	3 00	5 16	101 68	1035
" C <sub>1</sub>	Found	18 04	81 94	3 05	0 51	102 01	976
" C <sub>2</sub>	"	17 72	81 75		0 34	99 81	993
" "	Theory	17 83	80 24	3 14	0 51	101 72	987

Fraction A<sub>1</sub> is compared with a theoretical molecule consisting of eleven anhydroxyloses + one methoxyhexuronic acid anhydride + 6.57 per cent body X

Fractions A<sub>2</sub>, C<sub>1</sub>, and C<sub>2</sub> are compared with theoretical molecules consisting of six anhydroxyloses + one methoxyhexuronic acid anhydride + the amount of body X found by analysis

\* The reason the values in this column are greater than 100 per cent is because the oxygen and 2 of the hydrogen atoms of the methoxyl group were also included in the uronic acid anhydride and therefore were counted twice.

Methoxyl groups were determined qualitatively by Denigés' (7) method and quantitatively by the Zeisel (8) method. Von Fellenberg's (9) method of hydrolyzing the methoxyl groups proved that they were joined to the molecule by the ether linking and not by the ester or glucosidic linking. The small amounts of Fractions A<sub>2</sub> and C<sub>2</sub> were consumed by other tests before the presence of the methoxyl groups was investigated, consequently their analyses are incomplete in this particular.

The pentosan was determined by the phloroglucide method (10). After correcting the phloroglucide precipitate for that caused by the uronic acid, the pentosan was calculated as xylan.

The results of the analyses are shown in Table I. The sum of the constituents found is very close to 100 per cent in every case. If we assume the chemical composition of each hemicellulose to be that described by the theoretical values given in Table I, the analytical results check the theory in every particular. The analyses of the two fractions of Hemicellulose C are so similar as to indicate their identity. Fraction A<sub>2</sub> differs from the fractions of Hemicellulose C only in its high content of body X. This difference might have resulted through a splitting off of different amounts of this body during the extraction of the hemicellulose from the wood. It is not likely that the three simpler fractions are fragments resulting during extraction from the decomposition of Fraction A<sub>1</sub>. Such a conclusion would demand that five of the xylose units of Fraction A<sub>1</sub> were attached much less firmly than the other six. No experimental evidence supports such a conclusion. No fraction of hemicellulose reduced Fehling's solution, but when subjected to acid hydrolysis, all fractions very quickly became reducing. This fact supports the conclusion that the simpler fractions are not artifacts resulting from the decomposition of Fraction A<sub>1</sub>. It would appear, therefore, that two distinct hemicelluloses were extracted from mesquite wood with 5 per cent sodium hydroxide.

*Hydrolysis of Hemicelluloses and Separation of Hydrolytic Products*—In spite of their similarity, the four fractions of hemicellulose were hydrolyzed separately and the products studied. The conditions for hydrolysis chosen were those under which the hemicellulose of Norris and Preece (5) was completely hydrolyzed; that is, 1 per cent sulfuric acid at the boiling temperature. The heating was continued 6 rather than 4 hours. The hydrolysates were neutralized with barium hydroxide, freed from barium sulfate, and evaporated to a concentration which would produce a flocculent precipitate when poured slowly and with stirring into absolute alcohol. They were then precipitated with absolute alcohol. This procedure completely precipitated the barium salts contaminated with a little sugar and left the pure sugar dissolved in the alcohol.

Since all of the hemicelluloses were polyuronides, barium salts

should have been formed in each hydrolysate. This proved to be the case. The quantities obtained from the smaller Fractions A<sub>2</sub> and C<sub>2</sub> were insufficient to warrant further analysis so they were discarded but the barium salts from Fractions A<sub>1</sub> and C<sub>1</sub> were purified and analyzed. The results are shown in Table II. These analyses so closely check the theoretical values for the barium salt of a dixylonodimethoxyhexuronic acid as to indicate that the hemicellulose had been incompletely hydrolyzed. The barium content is consistently high throughout. This value was determined by igniting the salt in a platinum crucible and weighing the ash as barium carbonate. This ash was shown to contain barium

TABLE II  
*Analyses of Barium Salts*

	Uronic anhy- dride	Ba	Free aldehyde	CH <sub>2</sub> O	CH <sub>2</sub> O groups per uronic acid	Mol wt
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
Salt from Fraction A <sub>1</sub>	29 92	15 20	5 47	12 00	2 27	1176
“ “ “ C <sub>1</sub>	32 40	15 82	5 38	9 32	1 63	1086
Theory*	31 80	12 40	5 24	11 20	2 00	1107
Salt after hydrolysis in autoclave. . .	42 88	19 73	6 32	10 41	1 38	821
Theory†	43.19	16 86	7 12	7 61	1 00	915

\* For the barium salt of dixylonodimethoxyuronic acid.

† For the barium salt of xylonomethoxyuronic acid.

sulfate which had been held in solution by the salts during the process of purification.

These salts were combined with others obtained in the same manner and were subjected to hydrolysis in the autoclave at 1 atmosphere gage pressure in the presence of 1 per cent sulfuric acid for 5½ hours. After producing the barium salt and separating it from the sugar in the same manner as described above, the salt was purified and analyzed. The analysis of this salt is recorded in the fourth entry of Table II and corresponds closely to that of the barium salt of a methylated aldobionic acid. Although this shows that one more pentose unit has been removed by the treatment in the autoclave, the hydrolysis is still incomplete.

The methoxyl content of all of the barium salts is unaccountably

high. The analytical results pointed so accurately to but one methoxyl group per molecule of unhydrolyzed hemicellulose that one is tempted to attribute the higher content of the salts to the incomplete removal of ethanol from the salt samples before the Zeisel determination. Although the salt samples were dried over phosphorus pentoxide in a vacuum desiccator at room temperature, one sample was further dried in an Abderhalden vacuum drier at 110° after which it was analyzed for methoxyl content. It gave a result nearly identical with that obtained from the corresponding salt dried at room temperature. This discrepancy cannot be accounted for at this time. However, it does not vitiate the conclusion that the hemicellulose had been hydrolyzed to the stage of the aldobionic acid.

*Isolation and Identification of Xylose*—All of the alcoholic filtrates from which the barium salts were separated yielded xylose upon concentration. The sugar melted at 146–148°, gave  $[\alpha]_D = 18.5^\circ$ , and produced characteristic boat-shaped crystals of cadmium bromide-cadmium xylonate. The sugar solutions failed to give the diphenylhydrazone or the parabromophenylhydrazone of arabinose. The absence of methylpentoses was proved by the fact that the furfuralphloroglucide was completely insoluble in 95 per cent ethanol (11). The absence of galactose was established through failure to identify mucic acid after oxidation with nitric acid. The ease and completeness with which each syrup crystallized upon concentration was strong evidence that there was only one sugar present in the solution. The fact that the sums of the constituents found by analysis totaled so nearly 100 per cent confirms the conclusion that no sugar except xylose was present.

*Uronic Acid*—The negative mucic acid test proved the absence of galacturonic acid. Attempts to prepare potassium acid saccharate failed both when the salt was oxidized with nitric acid and when the oxidation was carried out according to the directions of Heidelberger and Goebel (12). Anderson and Otis (4) had difficulty in obtaining potassium acid saccharate from mesquite gum due to the fact that it was methylated but the larger quantities of the hydrolytic products available made possible the isolation of enough of the material for subsequent analysis. The position of the methoxyl group in the mesquite hemicellulose was narrowed down to the aldobionic acid. The difficulty encountered in the



identification of the uronic acid, viewed in the light of the experience of Anderson and Otis, suggested that the methoxyl group was attached directly to the uronic acid. The positive naphthoresorcinol test and the evolution of carbon dioxide when the hemicellulose was boiled with 12 per cent hydrochloric acid proved the presence of a uronic acid. Further than that we cannot report at this time.

#### CONCLUSIONS

When compared with other hemicelluloses reported in the literature, the hemicellulose extracted from mesquite wood by 5 per cent sodium hydroxide is relatively simple (1, 5, 6, 13, 14). Although the hemicellulose from mesquite wood was fractionated somewhat in the manner suggested by Norris and Preece, that is by a method successful in separating into their chemical entities two of the most complicated of the hemicelluloses, the mesquite hemicellulose gave fractions that were strikingly similar to each other. In spite of the fact that the methoxyl content of the two small fractions was not determined, all fractions should be classed as polyxylo-nomethoxyuronic acids. There was definite evidence of the presence of two distinct hemicelluloses but their difference lay mainly in the amount of xylose contained in the molecules.

The relation of these hemicelluloses to mesquite gum is slight. Both contain the methoxyuronic acid and it is possible that the uronic acid is the same. The xylose, on the other hand, is related in no simple way to the arabinose and galactose of the gum. This research indicates that the hemicellulose is not a precursor of the gum produced by the wood.

#### SUMMARY

1. The hemicellulose of mesquite wood was isolated and shown to be a mixture of two polyxylo-nomethoxyuronides.
2. The hemicelluloses were hydrolyzed and the hydrolytic products studied.
3. It was shown that there is no relationship between the hemicelluloses and the mesquite gum which would indicate that one is the precursor of the other.

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# CONTRIBUTION TO THE METHOD OF GAS ANALYSIS FOR RESPIRATION TRIALS

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It is shown in the present paper that the accuracy obtained in modern gas analysis (with errors of a few thousandths of 1 per cent) requires the consideration of secondary influences of temperature and partial pressure on the gas analysis and that also the distillation of water from the measuring pipette to the absorbing pipette has to be accounted for. A modification of the Haldane method of gas analysis is described by which errors resulting from changes in partial pressure and distillation of water from the measuring pipette are avoided.

## *Effect of Temperature Changes on Gas Solubility in KOH Solution*

In experiments carried out for the determination of the secondary influences of changes in temperature, the KOH in a Haldane apparatus has alternately been heated and cooled between temperatures of 13–37°. The average gain of gas in the measuring pipette per degree increase in temperature of the KOH was  $2.4 \times 10^{-3}$  per cent of the amount of gas (40 cc.) and the average loss (absorption by the cooling KOH) per degree decrease in temperature of the KOH was  $1.3 \times 10^{-3}$  per cent. These results show that the secondary influence of changes in temperature becomes significant when the temperature of the absorbing liquid changes 1° during one analysis.

## *Diffusion of O<sub>2</sub> from KOH Solution to N<sub>2</sub> in Gas Burette during Analysis*

In the use of the Haldane apparatus, it is customary, after complete absorption of CO<sub>2</sub> and O<sub>2</sub>, to pass the residual N<sub>2</sub> three

times over the KOH solution (Carpenter, Fox, and Sereque, 1929, p. 217). The KOH solution is saturated with  $O_2$  and  $N_2$  at their atmospheric tensions, and theoretically should yield some  $O_2$  to the  $N_2$  from the burette, and take from it some  $N_2$ . As the solu-

TABLE I  
Influence of Change in Partial Pressure on Result of Gas Analysis

Procedure	Reading	Decrease (absorbed by pyrogallate) in $10^{-3}$	Increase (given off by KOH) in $10^{-3}$
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
After absorption of $O_2$ (30 times in pyrogallate, including 3 rinsings of KOH capillary) and passing once over KOH	78 862		9
After passing over to KOH 10 times	78 871		11
" " " " " 10 "	78 882		9
" " " " " 10 "	78 891		10
" " " " " 10 "	78 901		
" " in pyrogallate 5 times and read without drawing into KOH	78 825	76	30
After passing into KOH <i>once</i>	78 855		
<i>Check</i>			
After absorption of $O_2$ (30 times in pyrogallate with 3 rinsings of KOH capillary) read <i>without</i> passing over KOH	79 451		19
After passing into KOH <i>once</i>	79 470		9
" " " " " 10 times	79 479		11
" " " " " 10 "	79 490		
" " " pyrogallate and read <i>without</i> passing into KOH	79 449	41	25
After passing into KOH <i>once</i>	79 476		8
" " " " " 10 times	79 484		
" " " pyrogallate and read <i>without</i> passing into KOH	79 430	54	18
After passing into KOH <i>once</i>	79 448		7
" " " " " 10 times	79 455		

bility of nitrogen is less than that of oxygen, the amount of nitrogen absorbed does not compensate the amount of oxygen given off by the KOH and the amount of gas in the measuring pipette should increase. The results in Table I indicate the magnitude of this effect obtained in a Haldane apparatus (Carpenter form).

Swift,<sup>1</sup> working in the Pennsylvania State College with a Carpenter form of the Haldane apparatus, had experiences similar to the ones described here and has related them to the same sources as we do. He overcame the difficulties by using the apparatus first for several CO<sub>2</sub> determinations, alone, and afterwards for the determinations of the sum of CO<sub>2</sub> + O<sub>2</sub>.

This principle of determining CO<sub>2</sub> and O<sub>2</sub> separately has been adopted by us. Two separate apparatus are used which are so constructed that the two determinations can be made simultaneously by one person.

*Influence of Distillation of Water from Measuring Pipette to  
Pyrogallol Solution*

The movement of the gas between the measuring pipette and the concentrated KOH-pyrogallate solution causes a distillation of water from the measuring pipette to the pyrogallol. Krogh (1920) has introduced an extra moistening pipette to avoid an error from this source. The author has constructed his apparatus so that the manometer may be used as a moistening device and decrease the distillation of water from the measuring pipette to the pyrogallol.

In 244 analyses carried out without the remoistening device, the average loss of liquid water from the measuring pipette was 0.76 c.mm. ( $1.9 \times 10^{-3}$  per cent of the air volume) per analysis. The amount of water was measured each morning after it had been allowed to gather in the stem overnight. For these analyses only about one-fifth of the amount of gas was driven back and forth between measuring pipette and pyrogallate, while four-fifths remained in the absorbing pipette. It is therefore to be expected that with the ordinary procedure of driving the total amount of gas back and forth the loss of liquid water is higher than the average loss of our 244 analyses. If the result of the analysis is given to 0.001 of 1 per cent, this loss of liquid water is thus to be considered. By the use of the remoistening device in 166 analyses, the loss of water was decreased to an average of 0.06 c.mm. ( $0.16 \times 10^{-3}$  per cent of the volume) per analysis. This amount is insignificant.

<sup>1</sup> Personal communication. Dr. Swift (1933) has now published his results on that subject and his method of gas analysis with the Carpenter form of the Haldane apparatus.

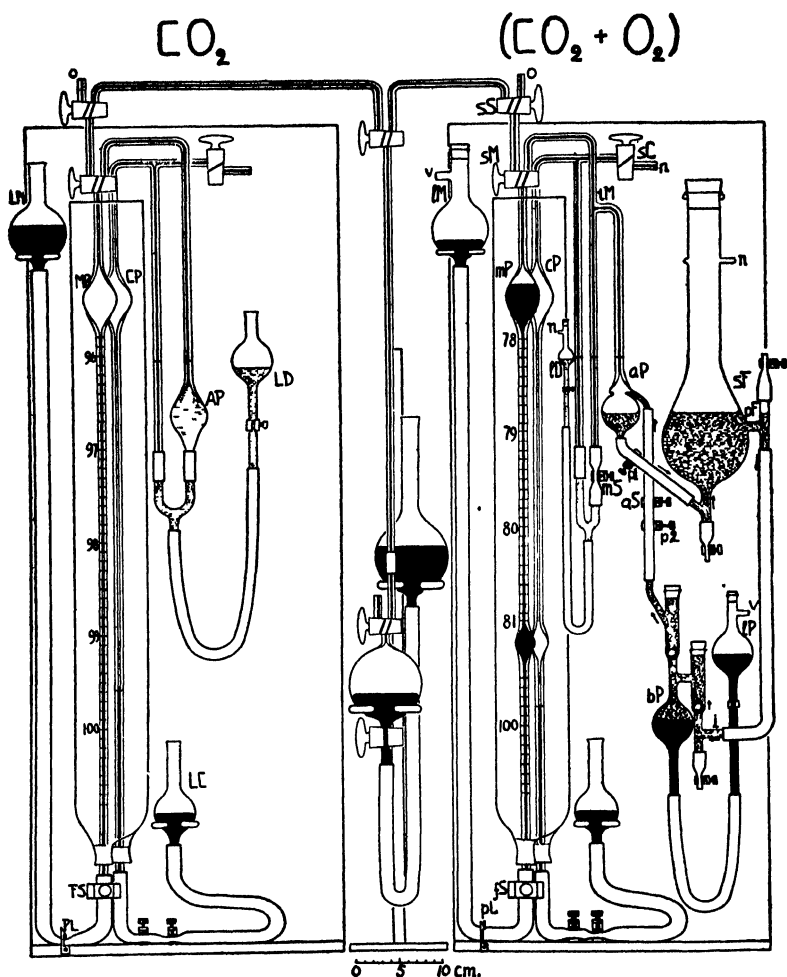


FIG. 1. A modified gas analysis apparatus in which AP, aP represent absorption pipettes; aS, screw clamp; bP, pump for pyrogallate circulation; CP, cP, compensating pipettes; FS, fS, pinch screws for fine adjustment of mercury level in measuring pipettes; i, inlet of circulating pump; LC, leveling bulb for mercury in compensating pipette; LD, lD, leveling bulbs for manometer liquid; LM, lM, leveling bulbs for mercury in measuring pipettes; LP, leveling bulb for mercury in pyrogallate circulating pump; MP, mP, measuring pipettes; mS, pinch screw; n, outlets of compensating

*Modified Gas Analysis Apparatus*

*Apparatus*—For the  $\text{CO}_2$  determination a true Haldane apparatus is used. The measuring pipette, *MP*, (see Fig. 1) of 40 cc. capacity has a capillary stem of 2.2 mm. bore graduated in per cent of the total volume from 96 to 100.2 per cent. The compensating pipette, *CP*, is without graduation but has a mark at the stem. The pipettes are mounted in a glass water jacket 8 cm. in diameter. The leveling bulb, *LM*, by means of which the mercury in the measuring pipette can be raised or lowered, has only one support which is so located that the mercury level in the pipette is at the lower end of the upper capillary when the leveling bulb is placed on the support. The connection between the leveling bulb and the measuring pipette is made by a thick walled nitrometer tubing and can be closed by means of a lever, *PL*, which pinches the rubber tube and is held in position by a catch as shown in Fig. 1. It is an advantage to close the connection between leveling bulb and measuring pipette after the sample is taken in because this avoids the oscillations of the mercury which delay a sharp reading. For that purpose the pinching lever is preferable to a ground in stop-cock because it avoids the rapid contamination of the mercury by stop-cock grease. The fine adjustment of the mercury meniscus in the measuring pipette is performed by means of a pinch screw, *FS*. Measuring pipette and absorbing pipette, *AP*, are one piece of glass so that the air sample after being measured does not come in contact with a rubber connection. The compensating pipette, *CP*, and its manometer capillary are also one piece of glass and the rubber connection to the absorbing pipette is below the level of the  $\text{KOH}$  in the manometer. This level can be adjusted to the mark at the capillary of the compensating pipette by means of the leveling

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pipette and leveling bulb of manometer and storage flask for pyrogallate. These outlets are connected to the inside of an air lock; *o*, outlet of measuring pipette; *p1* and *p2*, pinch-cocks; *pF*, side outlet; *PL*, *pL*, pinch levers closing connections between measuring pipettes and their leveling bulbs; *sC*, stop-cock of compensating pipette; *sF*, reservoir for pyrogallol; *sM*, stop-cock of measuring pipette; *sS*, stop-cock for intake and outlet of air sample; *tM*, capillary junction between manometer and absorbing pipette; *v*, outlets of leveling bulbs of measuring pipette and circulation pump connected to air pulsating system.



bulb, *LD*, as in any Haldane apparatus. The compensating pipette is open at the lower end and connected by means of a rubber tube to a leveling bulb, *LC*, containing mercury. The meniscus can be accurately adjusted to the mark on the capillary stem by means of two screw clamps. This modification of the compensating pipette has been found convenient for removing alkali solution which accidentally might have been sucked into the pipette; it is especially advantageous for moistening the entire inner surface of the pipette from time to time. This operation is necessary since readings during increases and decreases of the temperature in the water jacket led to the conclusion that the air in the compensating pipette may not be completely saturated with moisture even though there is enough liquid water in the capillary stem.

The sum of oxygen and carbon dioxide is determined in the apparatus shown on the right side of Fig. 1. The 40 cc. measuring pipette, *mP*, for the determination of the sum of oxygen and carbon dioxide has two bulbs connected by a 2.2 mm. capillary, graduated from 78 to 81 per cent of the total volume (40 cc.) and a lower stem graduated from 99.4 to 100.3 per cent. A similar compensating pipette, *cP*, is mounted beside the measuring pipette in the water jacket. The leveling bulbs, pinch lever, and adjusting screws are the same as already described for the carbon dioxide apparatus. In the case of the oxygen apparatus, it is impossible to have the air space in the compensating pipette always equal to that in the measuring pipette. If it is equal for the first reading, it is, of course, considerably different for the reading after the absorption of the oxygen, and *vice versa*. As both readings are of equal importance for the result, there is as much reason for having a lower bulb in the compensating pipette as for omitting this lower bulb.

For the oxygen apparatus, the true Haldane principle has been abandoned and an extra manometer has been installed. The rubber tube at the lower end of the manometer capillary of the measuring pipette is provided with a pinch screw, *mS*, by means of which the manometer liquid (water) can be raised to the capillary junction, *tM*, for moistening the air which passes by this junction when sucked from the pyrogallol pipette to the measuring pipette during the absorbing procedure. The absorbing pipette, *aP*, is fused to this junction as shown in Fig. 1 so that absorbing

pipette, manometer capillary, and measuring pipette are one piece of glass and the gas sample is in contact only with glass, mercury, water, and pyrogallol and not with rubber; furthermore, the entire surface of the space containing the gas sample is visible. The particular form of the absorbing pipette has been chosen in order to obtain a good absorbing surface and at the same time to avoid a considerable difference in the levels of the pyrogallol solution during absorption, which would mean a corresponding difference in pressure in the gas sample. The lower end of the absorbing pipette is connected to the lower outlet of the reservoir for pyrogallol, *sF*. A side outlet, *pF*, of this reservoir is connected to the intake, *i*, of a pump with two ball valves. A rubber tube connects the outlet of this pump to the upper side outlet of the absorbing pipette. The side outlet, *v*, of the leveling bulb, *lP*, of the mercury pump as well as the side outlet, *v*, from the leveling bulb for the measuring pipette is connected to a system in which suction and normal pressure are produced alternately in regular cycles of  $\frac{1}{2}$  to 1 minute's duration. The suction is produced in a closed 20 liter bottle of water placed on a shelf above a sink by syphoning the water out of it so that the air pressure inside the bottle is less than outside for a 70 to 100 cm. water level. By means of a 3-way stop-cock the outlets, *v*, of the gas analysis apparatus are alternately connected to the air in the suction bottle and opened to the outside air. The stop-cock is turned by a simple water wheel which is held by a catch in a certain position until the amount of water in one of the four cups of the wheel is sufficient to lift the catch and turn the wheel to the next position. The frequency of the pulsations in pressure can thus be controlled by regulating the velocity of the water flowing to the wheel. During suction, the mercury rises in the leveling bulb, *lP*, and pyrogallol is sucked through the lower ball valve into the bulb, *bP*. At the same time, the mercury rises in the leveling bulb of the measuring pipette, *lM*, causing one-fifth of the gas sample to flow back from the absorbing pipette to the measuring pipette. During the opening of the pulsating system to the outside air the mercury flows down from the bulb, *lP*, to the bulb, *bP*, forcing the pyrogallol contained in this bulb through the upper ball valve to the absorbing pipette, *aP*, where it flows down in a thin film covering almost the entire inside surface of the pipette. At the same time, the mercury

from the leveling bulb, *LM*, flows down to the measuring pipette, pressing the gas completely over into the absorbing pipette. By driving only one-fifth of the gas sample back and forth between absorbing pipette and measuring pipette, the stem error is reduced as well as the error due to the distillation of water from the measuring pipette to the pyrogallol.

The outlets, *n*, of the pyrogallol storage flask, of the manometer leveling bulb, and the compensating pipette<sup>2</sup> are connected to a water trap (not shown on Fig. 1). This arrangement keeps the air above the pyrogallol and the manometer liquid practically free of oxygen.

*Operation*—Fig. 1 shows the connection of the two apparatus to a gas sampling bulb between them. In order to introduce the air into the apparatus at the right the stop-cock, *sM*, is turned to make the connection to *sS*. As a considerable positive pressure is maintained in the sampling bulb, gas flows from this bulb to the measuring pipette as soon as the passage is opened by turning the stop-cock, although the leveling bulb, *LM*, remains in its position. When a small amount of air has entered the measuring pipette stop-cock *sS* is turned to the outside allowing this air from the measuring pipette to escape and the mercury to rise into the capillary above the bulb. By repeating this procedure three times all connections are washed out and filled with the gas of the sample. The measuring pipette is then again opened to the sampling bulb, the leveling bulb, *LM*, lowered, and gas is taken into the measuring pipette until the mercury meniscus is somewhat below the mark 100. The pinch lever, *pL*, is then pushed down to its catch and the leveling bulb can then be replaced in its support. The mercury meniscus in the compensating pipette is adjusted to the mark and stop-cock *sC* is closed. The manometer has been adjusted to the mark after the previous determination, when *sC* was opened to the outside. Stop-cock *sS* is then turned to outside so that gas from the sample can escape until the pressure in the measuring pipette is equal to the outside air pressure. If the outlet capillary, *o*, is sufficiently long, the opening can be made without risk that by difference in pressure the air of the sample might get mixed with outside air.

<sup>2</sup> During the analysis the compensating pipette is, of course, closed.

After the equilibrium of pressure has been established, the mercury meniscus is adjusted to the mark 100.000 and then stop-cock *sM* is turned so that the gas in the measuring pipette is connected to the manometer and to the absorbing pipette. Fine adjustment of the manometer level to the mark is made for the compensating pipette by means of the manometer leveling bulb, *lD*, and for the measuring pipette by means of the adjusting screw, *fS*. The level of the pyrogallol above the absorbing pipette should also be on the mark. However, no considerable adjustment should be necessary for the first reading of the air volume, because this would mean that the amount of nitrogen in the dead space is not the same at the beginning as at the end of the determination.

If, for example, the manometer liquid rises in the capillary leading to the measuring pipette after the gas sample in this pipette has been connected to the manometer, the mercury in the capillary stem of the measuring pipette must be raised in order to reestablish the equilibrium in pressure. For each unit of this raise (measured at the graduation of the capillary stem) 0.01 per cent of the amount of gas in the measuring pipette passes in to the air space of the capillary connection between measuring pipette and manometer. One-fifth of this 0.01 per cent of the gas is oxygen and will be absorbed subsequently. Since this oxygen is not a part of the air quantity measured in the pipette, its absorption causes an error of 0.002 per cent. The adjustment after connecting the measuring pipette to the manometer should, therefore, not exceed 0.01 per cent of the volume of the measuring pipette. After this adjustment, the volume of the gas in the measuring pipette is read and recorded. The manometer liquid is brought up to the junction, *tM*, by means of screw *mS*, and the connections of the absorbing pipette to the storage flask and the pyrogallol pump opened. The pinch lever, *pL*, is then lifted so that the mercury flows from the leveling bulb, *lM*, into the measuring pipette, driving the gas into the absorbing pipette. By turning a stop-cock, the leveling bulbs, *lM* and *lP*, are then connected simultaneously to the automatic pulsating system and the apparatus is left working for 20 minutes, during which time the analyst is free to make the carbon dioxide determinations. After that time, the connections, *v*, are opened to outside by turning the stop-cock which automatically closes the pulsating system. The air sample is

taken back to the measuring pipette by lowering the leveling bulb, *lM*, until the pyrogallol in the absorbing pipette almost reaches the mark at the capillary. The mercury connection between measuring pipette and leveling bulb is then shut by means of the pinch lever, *pL*. The two tubes connected to the absorbing pipette are closed by the pinch-cocks, *p1* and *p2*. By means of the screw clamp, *aS*, a small drop of water is then sucked down from the junction, *tM*, to the pyrogallol, moistening the capillary and clearing it of precipitates which may have accumulated. The meniscus of the pyrogallol is adjusted to the mark by using screw clamp *aS*. The screw clamp, *mS*, is then opened, the manometer adjusted to the mark at the capillary of the compensating pipette by means of the bulb, *lD*. Finally, the meniscus in the manometer capillary of the measuring pipette is adjusted to the mark by means of the screw clamp, *fS*, which influences the meniscus of the mercury in the measuring pipette. The volume is read and recorded, then the sample is driven over the pyrogallol again, and the pump operated for about 5 minutes. Another reading is taken and, if the result did not remain constant, the operation is repeated.

The volume of liquid water in the measuring pipette is read before the analyses are started in the morning after the water had time to drain from the bulb to the stem during the night. This volume is to be subtracted from the measurements of the oxygen determination.

The possible error and the waste of time and effort which may result in the combined apparatus by the effusion of oxygen from the potassium hydroxide solution after the absorption of the oxygen are avoided in our method. An error due to the distillation of water from the measuring pipette is eliminated. A further advantage of the new apparatus is the omission of a stop-cock between the pyrogallol pipette and the manometer. This stop-cock, according to Carpenter's method, has to be turned six times for the first absorption of oxygen and twice for each further control of the completeness of absorption (Carpenter, Lee, and Finerty, 1930, p. 15). Even with the best stop-cocks and the most carefully selected stop-cock grease, the turning of a stop-cock in connection with the gas sample always means a possibility of error and trouble. In our apparatus, no stop-cock in connection with

the gas sample has to be turned during the whole process after the initial reading has been made until the air is forced out again after completion of the analysis. It has already been mentioned that in this apparatus there is no rubber connection between the measuring pipette and the absorbing pipette and manometer. The necessary flexibility of the apparatus is obtained by rubber connections below the levels of the liquids.

A survey of the last twenty consecutive determinations which have been performed on the apparatus shows an average difference between two determinations on the same sample of 0.00175 per cent of  $\text{CO}_2$  and 0.0075 per cent for the sum of  $\text{CO}_2 + \text{O}_2$ . The time required for one complete analysis of a sample for  $\text{CO}_2$  and  $\text{O}_2$ , including the check determinations in both cases, is on the average 1 hour.

#### SUMMARY

In order to avoid errors from changes in the solubility of gases in the KOH solution used to absorb  $\text{CO}_2$ , the temperature of the solution should remain constant within  $1^\circ$  during one analysis.

The ordinary method of driving an air sample over KOH after the absorption of oxygen in the pyrogallate causes an error due to the effusion of oxygen from the KOH to the sample. This error is avoided by using two separate apparatus, one for the determination of  $\text{CO}_2$  and the other for the determination of  $\text{CO}_2 + \text{O}_2$ . By making use of an automatic pulsating system the two determinations are made simultaneously.

The apparatus for oxygen determination is so arranged that the distillation of water from the measuring pipette to the pyrogallate solution, which causes an error in the ordinary method, is decreased to an amount which is insignificant.

In both apparatus no stop-cock in connection with the air sample is to be turned after the first measurement of the volume. There is no rubber connection in contact with the air sample.

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## **SOME CONSIDERATIONS ON PRECISE ANALYSIS OF AIR FROM RESPIRATION CHAMBERS**

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In the preceding article Kleiber has discussed some conditions that may affect the accuracy of analyses of air of respiration chambers when the gas analysis apparatus devised in the Nutrition Laboratory is used. The many years of use of this gas analysis apparatus with various types of respiration chambers at the Nutrition Laboratory make it seem advisable to present at this time our own experience in connection with the problems raised by Kleiber's paper, the manuscript of which was kindly placed in our hands prior to publication.

The factors that may affect the analysis of atmospheric air or air from respiration chambers have previously been considered by Benedict (1), Krogh (2), Pickworth (3), and Vollmer (4). Benedict ((1) p. 75 ff.) used the Sonden (5) gas analysis apparatus and the other workers constructed special apparatus and devised routines to overcome the possible theoretical and practical difficulties in obtaining reliable results to within less than 0.005 per cent. Only the Sonden apparatus has ever been duplicated in any laboratory, but it has proved too fragile to be of practical use in most laboratories. When in the researches of the Nutrition Laboratory the necessity arose for a gas analysis apparatus in connection with open circuit respiration chambers, an apparatus was devised (6) that was based on the principles underlying the Haldane (7) apparatus. This apparatus has been in use for over 10 years and has undergone some modifications (8, 9), *which have been made principally with respect to shortening the time required to perform an analysis*. At the time that the apparatus was devised, it was recognized that the possibility of solution of oxygen and nitrogen in the two absorbing reagents and the possible errors resulting



from saturation and distillation of water might interfere with the desired accuracy of analysis; namely, on the average, 0.003 per cent.

During the course of an analysis with the gas analysis apparatus of the Nutrition Laboratory, distillation of water takes place. In view of this fact, it is our practise to saturate the gas after all of the oxygen has been removed, before making the final reading. The average amount of water that disappears is equivalent to 0.002+ per cent of the volume. This value is obtained by comparing the volume of water over the mercury at the beginning of a day's series with the volume of water found at the end and dividing by the number (17 or 18) of analyses made. However, a portion of this disappearance is due to the water vapor required to saturate the sample when it is drawn into the burette. In much of our work at the present time the samples are drawn into the sampler through a calcium chloride tube, in order to have them dry so that they may be analyzed 24 hours later, if necessary. There can be no doubt that some of the disappearance of water in the course of the day is due to the saturating of the dry samples drawn into the burette. Consequently, the size of the correction that should be made on the analyses due to the disappearance of water is uncertain. The maximum correction is the amount given above. It must be recalled that the use of the gas analysis apparatus in measurements of respiratory exchange is as a differential apparatus; that is, we determine the difference in composition between the air entering the respiration chamber and the air leaving it. It does not seem to us that there would be any particular gain in accuracy in subtracting so small a correction from *all* analyses, since the difference in composition between ingoing and outgoing air would be the same whether or not a constant absolute correction on all analyses was made.

The question of solubility of gases in liquids has been discussed in practically every article that deals with the theoretical possibilities of the causes of error in connection with gas analysis. We have sought over and over again to secure evidences of the solution of gases in liquids in connection with our use of gas analysis apparatus over a period for more than 10 years and thus far have not been able to obtain any consistent evidence that solution takes place to a measurable degree or that the solubility of gases in the

reagents used interferes with the accuracy desired in the investigations in progress. Theoretically, the severest test of the possibility of diffusion of gases would be one in which the gases on two sides of the reagent were different from one another, for example, pure nitrogen on one side and oxygen-rich air on the other. We have recently made a series of tests to determine whether under such favorable conditions for diffusion of gases through the potassium hydroxide solution diffusion would take place to a measurable extent. This was done by filling a toy balloon with oxygen and attaching it to the opening of the potassium hydroxide leveling bulb connected with a reservoir of potassium hydroxide for the absorption of carbon dioxide. Preliminary tests with such a procedure showed us that the mixture above the potassium hydroxide had approximately 60 per cent of oxygen. If there were a diffusion through the potassium hydroxide, three effects might take place: (1) The volume of oxygen passing into the interior of the apparatus might be equaled by the volume of nitrogen diffusing out through the potassium hydroxide; (2) the volume in the inside of the apparatus might increase owing to an actual added amount of oxygen above the nitrogen already present in the apparatus; (3) nitrogen might go out of the apparatus without a compensating inward diffusion of oxygen. In the first case there would be no change in readings when the gas was passed over the potassium hydroxide. In the second case there would be an increase in volume when the gas was passed over the potassium hydroxide and a subsequent decrease when it was passed into the pyrogallate solution, and in the third case there would be a decrease in volume when the gas was passed into the potassium hydroxide. The results of such a test are shown in Table I. The first set of readings was carried out for the purpose of demonstrating that the oxygen was completely absorbed. As usual, after the gas was passed into the pyrogallate solution, it was saturated with water vapor by being passed three times into the 20 per cent potassium hydroxide before reading. Subsequently, it was passed five times into the potassium hydroxide and this procedure repeated three times. Then the gas was passed into the potassium pyrogallate in order to ascertain whether any oxygen had passed into the gas. Subsequently, it was passed into the potassium hydroxide ten times, and this operation was repeated twice. Finally, the gas

was again passed into the pyrogallate, and two readings were made with this procedure. The maximum range of readings extending over a period of 40 minutes was 0.004, and there is no consistent evidence that there was any diffusion of gases either out of the apparatus or into it. The final reading is 0.002 less than the first reading. These tests as well as others do not furnish any consistent evidence of the diffusion of gases through the potassium hydroxide solution.

Two methods have been used to test the accuracy of performance with the apparatus. One is the frequent analysis of outdoor

TABLE I

*Effect of High Oxygen (60 Per Cent) in Leveling Bulb of Potassium Hydroxide Pipette on Readings of Gas Analysis Apparatus*

						Reading
						<i>per cent</i>
After removal of O <sub>2</sub>						79 289 .
"	3	times into pyrogallate,	3	times into KOH		79 286
"	3	"	"	3	" " "	79 286
"	3	"	"	3	" " "	79 287
"	5	"	"	KOH		79 287
"	5	"	"	"		79 289
"	5	"	"	"		79 289
"	3	"	"	pyrogallate, 3 times into KOH		79 285
"	3	"	"	3	" " "	79 286
"	10	"	"	KOH		79 289
"	10	"	"	"		79 289
"	3	"	"	pyrogallate, 3 times into KOH		79 288
"	3	"	"	3	" " "	79 287

air, which was found by Benedict (1) to be practically constant in composition both with respect to carbon dioxide and to oxygen. It has been the custom in our work to make an analysis of outdoor air nearly every day that the apparatus is used and sometimes more than once during the day. Examples of several series of analyses of outdoor air are given in Table II. These series were all made as routine controls for investigations in progress. Series 2 and 3 were accumulated in connection with two researches (10, 11) on the effects of sugars on the respiratory exchange of man. Series 4 and 5 are controls made in Baltimore, each operator using

a different apparatus. All of the series, with the exception of that of M. T. S., include *every* analysis made during the periods of time covered by the respective series.

TABLE II  
*Analyses of Outdoor Air*

Series No.	Apparatus No.	Analysts	No. of analyses	Average CO <sub>2</sub> per cent	Standard deviation	Average O <sub>2</sub> per cent	Standard deviation
1	13, 20	E. L. F.	176	0.031	0.0016	20.940	0.0031
2	3, 20, 38, 112	4	76	0.031	0.0017	20.940	0.0038
3	18, 41, 139	6	68	0.031	0.0014	20.940	0.0036
4	20	M. T. S.	92	0.033	0.0009	20.940	0.0039
5	8	K. K.	48	0.031	0.0010	20.939	0.0034

A second proof of the accuracy of the apparatus is the determination of the respiratory quotient in air samples from a chamber in which either alcohol or acetone is burning. The results of three series of such checks follow.

Series No.	Analyst	Location	No. of analyses	Average r.q.	Standard deviation
1	K. K.	Boston	12	0.664	0.0038
2	"	Baltimore	24	0.665	0.0051
3	G. L.	Boston	26	0.749	0.0034

Series 1 was made by K. K. in December, 1932, while learning to operate the apparatus. Series 2 consists of routine alcohol checks made during experiments in progress in Baltimore. The series with acetone by G. L. was accumulated as controls in connection with an investigation in progress on the effects of sugars on the respiratory quotients of animals.

The attempts of various workers to overcome the small errors that are theoretically possible in gas analysis have met with only partial success and have resulted in the construction of extremely complicated apparatus to be used under rigid conditions of temperature control. These apparatus are impractical because they are not easily duplicated and are not at all transportable.

In view of the various series of analyses of outdoor air and of

the determinations of respiratory quotients of burning alcohol or acetone we shall continue to use this apparatus in this laboratory and cooperating laboratories until it is either modified or another is devised that is more accurate, speedier, and at the same time a practical apparatus, easily duplicated and transportable without danger of breakage. The routine now used has been subjected to so many trials and variations without appreciable improvement that we shall continue to use the same procedure until a better one is devised. We would strongly urge that when an investigator believes he has a superior apparatus he submit evidence that his apparatus and technique will give constant results with outdoor air and with air from burning alcohol or acetone within the range of accuracy demanded in the investigations for which the apparatus is designed. Concrete examples of accuracy of repeated determinations of outdoor air or of checks with substances such as alcohol and acetone for which one can calculate the theoretical ratios of carbon dioxide given off to oxygen absorbed are missing in most publications on gas analysis.

*Addendum*—Since this article was submitted for publication a contribution by Swift (12) has appeared in which the two questions of absorption of water by the potassium pyrogallate and the solution of gases in the reagents are considered. Swift has found a much greater disappearance of water during the procedure of analysis than has been noted either by Kleiber or by us. With regard to the number of analyses which can be made with one filling of the pyrogallate pipette containing 450 cc., we have set 100 (9) as a very conservative figure in order that at no time would we be obliged to change the solution in the course of a series of analyses when our time was limited for the completion of work in progress. To be obliged to change the pyrogallate solution in the midst of a group of analyses would result in such a delay that it is far more economical for us to change the solution at a convenient time than it would be for us to continue the use of the solution until nearly or entirely exhausted. We have records of several fillings of the pyrogallate pipette by our method in which over 200 analyses have been made and the solution was still efficient. After 100 or more analyses have been made the solution tends to become muddy with aggregations of solid particles which interfere with the setting of the level. The statement that "the use of a satisfactory respiratory quotient in testing the tightness of a metabolism apparatus is inadequate" because a leak of room air into it affects the quotient but little is correct so far as open circuit apparatus is concerned, but does not apply to closed circuit apparatus. If this statement is supposed to apply to gas analysis apparatus, it is also true, but the use of duplicate analyses of the same sample of air from burning

acetone or alcohol will serve as a check on the tightness of the gas analysis apparatus, since it is highly improbable that the leakage of room air into the apparatus would be exactly the same in each analysis.

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## **STUDIES OF THE PEPTIDES OF TRIVALENT AMINO ACIDS**

### **III. THE APPARENT DISSOCIATION CONSTANTS, FREE ENERGY CHANGES, AND HEATS OF IONIZATION OF PEPTIDES INVOLVING ARGININE, HISTIDINE, LYSINE, TYROSINE, AND ASPARTIC AND GLUTAMIC ACIDS, AND THE BEHAVIOR OF LYSINE PEPTIDES TOWARD NITROUS ACID\***

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#### **INTRODUCTION**

In the study of complex biological systems it is often desirable that their behavior be studied by comparison with the analogous behavior of simpler models of known structure and composition. In the case of the proteins, evidence collected on their simplest units, the amino acids and their peptide combinations, helps to elucidate certain characteristics of the larger molecules.<sup>1</sup> Systematic data are being collected in this laboratory (3-6) on the electrochemical behavior of the amino acids, which in many respects parallels the amphoteric behavior of the natural protein. In the present series of studies (7, 8), the attempt is made to compare the dissociation values found in the proteins with the values deter-

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† The compounds described in this paper, with the exception of glycylproline, were prepared by the author while National Research Council Fellow in Biochemistry at the Kaiser Wilhelm Institute, Dresden, 1931-32. The peptide, glycylproline, was kindly furnished by Professor M. Bergmann of the above Institute.

<sup>1</sup> For an extensive discussion and summary of this problem, see Cohn (1, 2).



mined from synthetic peptides containing those free groups not involved in peptide linkage, and responsible for the protein-ionizing capacity. Such groups are the  $\epsilon$ -amino group of lysine, the imidazole ring in histidine, the free carboxyl group in the dibasic aspartic and glutamic acids, the guanidine nucleus of arginine, and the hydroxyphenyl ring of tyrosine. Alterations in the values of the dissociation constants of these groups when found in the amino acids are decidedly marked when these acids are combined in peptide linkage. Further lengthening of the chain beyond the dipeptide stage, as Stiasny and Scotti (9) indicated, produces only very much smaller changes in the values of the amino and the carboxyl groups. If the protein therefore is to be considered as composed largely of long peptide chains, and if any forces other than those due to primary valency are neglected, the protein dissociation values may best be compared with those found in the dipeptides. The mutual effect of the free ionizing groups crowded together in the smaller synthetic molecules will reflect the analogous behavior of such groups in the protein.

In previous papers (7, 8) this effect was studied in such peptides as histidylhistidine, aspartylaspartic acid, and tyrosyltryptophan. It was found that, while the interposition of the peptide linkage has always the effect of considerably weakening the amino and the carboxyl groups, this weakening may be somewhat modified by the presence of other free ionizing groups in the molecule. The shifts in the values of these groups are less than those of the amino or the carboxyl group, and their direction toward more acid or alkaline range will depend upon the nature of the groups in proximity. Thus the basic imidazole group will be slightly more acid than in histidine when found next to the basic amino group in the peptide (7); on the other hand, the acid hydroxyphenyl ring in the peptide, when the carboxyl group is in proximity, will be slightly more alkaline than in tyrosine (8). These general phenomena are already familiar from the work of Ostwald (10) and Bredig (11) and are further exemplified in the results on the diverse substances described below. In general, it may be stated that the effect of these extra ionizing groups is to increase the dissociation of the carboxyl group whether in the amino acid or in the peptide. The acidic property of the carboxyl group in tyrosine, histidine, and arginine is uniformly higher than in glycine (1) and this strengthen-

ing effect, combatted by the weakening influence of the peptide linkage, is carried over into the peptide molecule. Not only the groups peculiar to these acids, but the presence of additional amino groups will likewise cause an increased acid dissociation, and, as shown by the author (12) and by Schmidt, Appleman, and Kirk (5), this increase will be inversely proportional to the distance between the dissociating groups.

In the present communication, the dissociation values of peptides containing several free groups will be reported, as well as their free energy changes and heats of ionization. Data on the heats of ionization of several complex amino acids have been obtained by Schmidt, Kirk, and Appleman (4) and by Branch and Miyamoto (13). Miyamoto and Schmidt (6) have reported the apparent free energy and entropy changes due to ionization of the amino acids. The present study is concerned partly with the dissociation of peptides containing the guanidine nucleus, the oxyphenyl and imidazole rings, and the free amino and carboxyl groups, as reflected in the titrations of phenylalanylarginine, tyrosylarginine, aspartyltyrosine, histidylglycine, lysylglutamic acid, and lysyllysine. For purposes of comparison the titrations of glycylglycine and of phenylalanylglycine have been redetermined and included. Potentiometric measurements were made at 25° and at 0° and the heats of ionization were calculated from the results obtained at these temperatures. While all the peptides hitherto studied contain the usual substituted acid amide binding between amino acids, the peptide, glycylproline, is unique in that its peptide linkage contains no hydrogen. It was thought to be of interest to include the titration of the peptide of this interesting and widely occurring imino acid.

It has been shown by Dunn and Schmidt (14) that the rate of deamination of variously substituted amino acids with nitrous acid decreases with increasing distance between the amino and the carboxyl groups, and the same authors and Schmidt (15) have demonstrated that this rate is likewise a function of the temperature. In the present paper will be reported the rate of deamination of peptides of lysine as reflected in the behavior at constant temperature of lysyllysine, lysylglutamic acid, and lysylhistidine toward nitrous acid. It will be shown that this rate is about double that characteristic of lysine.

## EXPERIMENTAL

*Preparation of Materials*—The peptides, phenylalanylarginine, tyrosylarginine, and histidylglycine, were prepared respectively according to the general azlactone methods of Bergmann and Köster (16), Bergmann, Zervas, and du Vigneaud (17), and Bergmann and Zervas (18). The arginine for the synthesis of the arginine-containing peptides had been isolated from gelatin by the benzaldehyde procedure of Bergmann and Zervas (19). Aspartyltyrosine was prepared according to the new carbobenzoxy method of Bergmann and Zervas (20). Lysylglutamic acid and lysylhistidine were identical samples reported by Bergmann, Zervas, and Greenstein (21). Lysine anhydride, phenylalanylglycine, and glycyglycine were prepared respectively according to the methods of Fischer and Suzuki (22), Fischer and Blank (23), and Fischer and Forneau (24). The lysine dihydrochloride employed for the determination of the rate of deamination by nitrous acid was isolated from hemoglobin after the procedure of Vickery and Leavenworth (25). All of these substances had been recrystallized and their melting points and elementary analyses checked. Glycylproline, supplied through the kindness of Professor Max Bergmann, was part of the identical sample reported by Bergmann, Zervas, Leinert, and Schleich (26).

*Procedure*

In nearly all cases a 0.02 M solution of the peptide in water was prepared, appropriate aliquots were removed and treated with either 0.040 M or 0.080 M HCl or NaOH, and the resulting solution was made up to such volume that the end-concentration of ampholyte was 0.01 M. For the preparation of a 0.02 M solution of lysyllysine from lysine anhydride dihydrochloride, a sample of 0.1645 gm. of the latter substance was dissolved in 7.5 cc. of N NaOH and allowed to stand for 24 hours at room temperature. At the expiration of this time, 7.5 cc. of N HCl were added, and the mixture was made up to 25 cc. with water. As shown previously (7, 8), the diketopiperazine is quantitatively split under these conditions and this is further confirmed in the case of this substance by the amino nitrogen and the titration data.

Potentiometric readings were made at 25° and at 0°, the hydrogen and calomel electrodes being kept at the former tempera-

ture by a controlled air chamber, and at the latter temperature by an ice water bath. The 0.1 N KCl calomel electrode was standardized at 25° with 0.1 N HCl, Scatchard's value for  $\gamma_{H^+}$  of 0.841 being used (27). For the determination of the value of the calomel electrode at 0°, a solution of 0.01 N HCl plus 0.09 N KCl was employed. The value of  $\gamma_{H^+}$  of this solution was taken as 0.907 (27). The negative logarithm of the dissociation constant of water was accepted at 25° as 13.998 and at 0° as 14.943. ▲ The activity

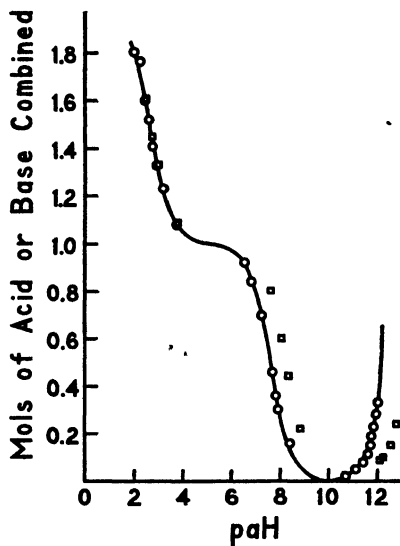


FIG. 1. Titration curve of phenylalanylarginine based on the constants:  $pK_1' = 2.66$ ,  $pK_2' = 7.57$ ,  $pK_3' = 12.40$ . ○ indicates experimental points at 25° and □ at 0°.

coefficients of  $H^+$  and  $OH^-$  employed for the calculation of the acid and the base bound by the ampholyte were assumed identical with those with respect to pure HCl and NaOH solutions respectively, and were taken in part from the data of Lewis and Randall (28) and in part from the results of blanks carried out with pure water.

For the purpose of illustration, the titration curve of phenylalanylarginine is given in Fig. 1. The ordinate represents the acid or base bound per mol of peptide, the abscissa the pH. The

experimental points are indicated at 25° by circles and at 0° by squares. The curve is theoretical and is calculated from the titration constants chosen.

The dissociation of each free group is represented by  $\alpha$  in the mass action expression for the behavior of a monovalent acid or base:

$$G' = H (\alpha/(1-\alpha)) \text{ or } pG' = p\alpha H + \log ((1-\alpha)/\alpha)$$

where  $G'$  is the apparent titration constant<sup>2</sup> related to the classical apparent dissociation constant  $K'$  by expressions first developed by Simms (29). Inasmuch as the corresponding values of  $G'$  and  $K'$  for each group differ from each other by an amount less than the experimental error in determining such constants, it seemed preferable to retain only the value for  $K'$  throughout this communication.

The apparent dissociation constant  $pK'$  of each isolated group may then be calculated from the  $p\alpha H$  at which that group is half neutralized. When, however, two or more groups dissociate simultaneously within the same  $p\alpha H$  range, then the  $pK'$  values of these groups must be so chosen that at each  $p\alpha H$  value that the experiments reveal, the sum of the dissociation stages,  $\Sigma\alpha$  of all such groups, will yield the mols of acid or base combined;  $\Sigma\alpha = x$ .<sup>3</sup>

For the calculation of the free energy changes due to ionization, the following relation, similar to that employed by Miyamoto and Schmidt (6), was used  $(\Delta F^0)' = -RT \ln K'$  where  $(\Delta F^0)'$  is a constant at any one temperature and  $K'$  is the apparent dissociation constant. At constant temperature, the change in free energy is a measure of the maximum work performed by the system between the standard and the actual states. In the above expression, the change in free energy represents the work involved in each step of ionization (represented by  $K'$ ) and will be proportional to the algebraic sum of the various effects due to the nature of the dissociating group, the influence of other substituents upon this group, and the electrostatic forces existing among all other groups in the

<sup>2</sup> Inasmuch as in the above equation the first member on the right-hand side is expressed as activity and the second member as concentration, the constant  $pG'$  is of a hybrid or apparent character

<sup>3</sup> For definition of  $\alpha$ , the acid or base combined per mol of ampholyte, refer to Paper I of this series (7).

molecule. These effects have been estimated by Bjerrum (30) and by Simms (31) in the case of the isomeric divalent acids. It is doubtful whether similar considerations could be applied to the complex ampholytes, and the values of  $(\Delta F^0)'$  listed in Table I are limited to the actual numerical values found for  $K'$ . Miyamoto and Schmidt (6) have shown that no appreciable error was introduced into the calculations of the free energy when the values for the apparent dissociation constants were used in place of the true dissociation constants.<sup>4</sup>

For the purpose of calculating the apparent heats of ionization, the equation

$$-(\Delta H)' = \frac{d(R \ln K')}{d(1/T)} = 14,940 \Delta pK'$$

was employed, according to the assumptions previously made by Schmidt, Kirk, and Appleman (4) and by Branch and Miyamoto (13).

*Amino Nitrogen Determinations*—The usual Van Slyke apparatus was employed in a room whose temperature varied from 22° by not more than  $\pm 1^\circ$ . The solutions of lysine and of the peptides were so prepared that a given aliquot of 2 cc. would yield about 2 cc. of nitrogen gas. Time intervals of shaking from 1 minute in duration upwards were employed. Customary blank determinations were made.

#### DISCUSSION

In Table I are listed the dissociation values and heats of ionization of the peptides studied as well as some data on amino acids reported by Schmidt, Kirk, and Appleman (4).

It is apparent that the free groups of the peptides, while usually dissociating at ranges far removed from those of the same groups in amino acids, nevertheless yield similar values for the heat of

<sup>4</sup> The transformation of  $K'$ , the so called apparent constant, to  $K$ , the thermodynamically defined constant when all members in the reaction at equilibrium are expressed as activities, consists essentially in multiplying  $K'$  by the ratio of the activity coefficients of the charged and uncharged forms of the ampholyte. For most practical purposes, the activity coefficient of the non-ionized molecule may be taken as unity. The activity coefficients of certain amino acid ions derived from freezing point data have been reported by Hoskins, Randall, and Schmidt (32).

ionization. That strongly acid groups have very small heats of ionization, whereas strongly basic groups possess large heat values in the neighborhood of 10,000 to 12,000 calories, has already been pointed out among others by Kolthoff (33), Ebert (34), and Meyerhof (35).<sup>5</sup> The question of the influence of temperature on dissociation and the zwitter ion constitution of amphoteric electrolytes has been summarized by Cohn (1). It has been shown by Schmidt, Kirk, and Appleman (4) that the strongly basic groups such as the amino and guanidine groups possess values of heat of ionization in the neighborhood of 10,000 to 12,000 calories, whereas the weakly basic imidazole ring in histidine yields a value considerably lower than this. Similar values are obtained with peptides containing these groups (Table I). Again, the strongly acid carboxyl group has a negligible heat of ionization both in the amino acids and in the peptides, whereas the very weakly acid hydroxyphenyl ring yields a value intermediate between those of full basic and full acid heat values, namely in the neighborhood of 6000 calories (Table I). It is probable, therefore, that the dissociation of the same groups in the larger protein molecule will behave similarly with temperature and some evidence in this direction has been presented by Pertzoff and Carpenter (36).

The values of the free energy change ( $\Delta F^0$ )', corresponding to each step in ionization described by  $K'$ , will be proportional to the actual numerical value of  $K'$ . Consequently, these values will be somewhat different in the peptides and in the amino acids. The work involved in the ionization of the carboxyl groups of the monoaminomonocarboxylic acids, as shown by Miyamoto and Schmidt (6), is of the order of 3000 calories, while that of the amino groups of the same acids is about 13,000 calories. An intermediate value is obtained for the imidazole ring of histidine, and the highest value of 17,000 calories is given by the guanidine

<sup>5</sup> It is therefore, possible, by estimating the magnitude of the apparent heat of ionization, to predict the nature of the group which is dissociating. This is clearly of advantage in dealing with the complex peptides containing crowded free groups which may have, as in the case of tyrosylarginine, several groups simultaneously dissociating within a rather narrow pH range. Those organic radicals which are either weakly acid or weakly basic, such as the hydroxyphenyl and the imidazole rings respectively, will yield intermediate heat values and by this criterion may be readily identified.

nucleus of arginine. In the case of the peptides (Table I) the work involved in the ionization of a carboxyl group will be slightly greater, that of an amino group will be considerably less, than the work involved in the ionization of the same groups in the amino acids. The extent of these differences will vary with the nature of other substituents in the molecule. When extra basic substituents are present, the work involved in the carboxyl dissociation will be still less than when no such groups are present. In the case of lysyllysine, this energy will be even less than that involved in the ionization of the carboxyl group in lysine (Table I).

In a consideration of the free energy change as a function of the temperature, and if the Gibbs-Helmholtz equation is employed

$$(\Delta F^0)' = (\Delta H)' + T (d(\Delta F^0)'/dT) \cdot$$

it is evident that in the case of the carboxyl group dissociation where  $(\Delta H)'$  is negligible or zero, the free energy change will be proportional to the absolute temperature. On inspection of the values for  $(\Delta F^0)'$  corresponding to the carboxyl groups and listed in Table I, it is observed that these values increase by 300 to 400 calories between 0–25°. Furthermore, if the assumption be made that the change in free energy varies linearly with the temperature, values of  $(\Delta F^0)'$  of the order of magnitude of 3000 to 4000 calories are obtained, which are characteristic of the free energy change involved in the ionization of the carboxyl group. Conversely, values for  $(\Delta H)'$  of 500 to 1000 calories may be calculated from the known values of  $(\Delta F^0)'$  and its temperature coefficient for the carboxyl group.<sup>6</sup> On the other hand, when the change in free energy is independent of the temperature, that is when  $d(\Delta F^0)'/dT = 0$ , then the change in free energy due to ionization should equal the heat of ionization. This relation is closely followed in the case of the amino and imidazole groups of all the compounds listed in Table I. The values of  $(\Delta F^0)'$  corresponding to the imidazole and amino groups are practically constant between 0–25° and according to the above equation the values of  $(\Delta F^0)'$  and  $(\Delta H)'$

<sup>6</sup> Since  $(\Delta H)'$  values are proportional to the difference in the values of  $pK'$ , any errors in the determination of the latter may be magnified in the values of  $(\Delta H)'$ . An error in  $\Delta pK'$  of 0.05 unit will cause a deviation in  $(\Delta H)'$  of 750 calories. In contrast, an error in  $pK'$  of 0.05 unit will cause an error in  $(\Delta F^0)'$  of about 65 calories.



# 612 Peptides of Trivalent Amino Acids. III

TABLE I  
Ionization and Energy Values of Peptides

	Temperature	Group*	pK'	$\Delta pK'$	( $\Delta H$ )'	( $\Delta F^\circ$ )'	pI
	°C				calories	calories	
Phenylalanylarginine	0	Carboxyl	2.69			3,300	10.72
	25		2.66	0.03	450	3,700	9.99
	0	Amino	8.25			10,300	
	25		7.57	0.68	10,150	10,300	
	0	Guanidine	13.20			16,600	
Arginine (Schmidt et al.)	25		12.40	0.80	11,950	17,000	
	0	"	13.31			16,700	11.55
	25		12.48	0.83	12,400	17,100	10.76
Phenylalanylglycine†	0	Carboxyl	3.14			3,900	5.76
	25		3.10	0.04	680	4,300	5.40
	0	Amino	8.38			10,500	
	25		7.71	0.67	10,000	10,500	
Tyrosylarginine	0	Carboxyl	2.65			3,300	8.93
	25		2.65	0	0	3,600	8.38
	0	Amino	8.09			10,100	
	25		7.39	0.70	10,500	10,100	
	0	Oxyphenyl	9.76			12,200	
	25		9.36	0.40	6,000	12,800	
	0	Guanidine	12.50			15,700	
	25		11.62	0.88	13,000	15,900	
Aspartyltyrosine	0	Carboxyl	2.18			2,700	2.88
	25		2.13	0.05	750	3,000	2.85
	0	"	3.57			4,500	
	25		3.57	0	0	4,900	
	0	Amino	9.60			12,100	
	25		8.92	0.68	10,160	12,200	
	0	Oxyphenyl	10.65			13,400	
	25		10.23	0.42	6,200	14,000	
Histidylglycine	0	Carboxyl	2.42			3,000	7.42
	25		2.40	0.02	300	3,300	6.81
	0	Imidazole	6.30			7,900	
	25		5.80	0.50	7,500	7,900	
	0	Amino	8.54			10,700	
	25		7.82	0.72	10,800	10,700	
Histidine (Schmidt et al.)	0	Imidazole	6.50			8,100	8.13
	25		6.04	0.46	6,900	8,300	7.58
Glycylglycine†	0	Carboxyl	3.16			3,900	6.00
	25		3.12	0.04	680	4,300	5.65
	0	Amino	8.86			11,100	
	25		8.17	0.69	10,300	11,200	

TABLE I—*Concluded*

	Temperature	Group*	pK'	$\Delta pK'$	$(\Delta H)'$	$(\Delta F^\circ)'$	pI
	$^{\circ}C$				calories	calories	
Lysylglutamic acid	0	Carboxyl	2.98			3,700	6.45
	25		2.93	0.05	750	4,000	6.10
	0	"	4.47			5,600	
	25		4.47	0	0	6,100	
	0	Amino	8.45			10,600	
	25		7.75	0.70	10,500	10,600	
	0	"	11.30			14,200	
	25		10.50	0.80	11,950	14,400	
Lysyllysine	0	Carboxyl	2.10			2,600	10.87
	25		1.95	0.15	2,000	2,700	10.04
	0	Amino	9.02			11,300	
	25		8.17	0.85	12,700	11,200	
	0	"	10.21			12,800	
	25		9.45	0.76	11,350	12,900	
	0	"	11.52			14,500	
	25		10.63	0.89	13,300	14,600	
Lysine (Schmidt et al.)	0	Carboxyl	2.20			2,700	10.56
	25		2.18	0.02	300	3,000	9.74
	0	Amino	9.81			12,300	
	25		8.95	0.86	12,800	12,300	
	0	"	11.31			14,200	
	25		10.53	0.78	11,600	14,400	
Glycylproline	25	Carboxyl	2.84			3,900	5.73
		Amino	8.62			11,800	

\* The constants calculated are assigned to specific groups on the basis of the zwitter ion constitution of amphoteric electrolytes.

† Values at 25° for phenylalanyl-glycine were reported by Mitchell and Greenstein (37) as  $pK_1' = 2.05$ ,  $pK_2' = 8.02$ . It is believed that these results are in error owing to the use of impure material. These investigators reported an erratic potential (p. 259) which was undoubtedly due to the presence of cinnamoyl derivatives and which would account for the electrode poisoning described. After several recrystallizations of the peptide from hot water, no difficulties were encountered.

‡ Dissociation values for glycylglycine at these two temperatures are comparable with those determined by Branch and Miyamoto for this compound (13). They found  $pK_1' = 3.02$  at 0° and  $pK_1' = 3.06$  at 25°, with  $(\Delta H)' = -600$  calories. The basic constant was reported as  $pK_2' = 8.80$  at 0° and 8.13 at 25° with  $(\Delta H)' = 10,000$  calories.

should be about equal. Within the experimental error, these values may be considered equal (Table I).<sup>7</sup>

The temperature coefficient of  $(\Delta F^\circ)'$  in the case of the carboxyl, hydroxyphenyl, and guanidine groups, is always positive and of the same magnitude. The behavior of the latter two groups cannot be described in terms of the Gibbs-Helmholtz equation. It is possible that in the instance of the guanidine group the very considerable errors resulting in the determination of its dissociation constant in regions of high alkalinity are a contributing factor.

While the heat of ionization appears to be characteristic of the nature of the dissociating group and independent of the constitution of the ampholyte molecule, the value of the titration constant relating to any particular group is a function both of that group's position in the molecule and the presence of adjoining groups. In all cases, the effect of the introduction of the peptide linkage between amino acids is simultaneously to weaken and narrow the range of dissociation of the free groups over that of the same groups when found in the amino acids. Modifying this weakening effect of the acid amide linkage on the carboxyl and amino groups is the influence of neighboring substituted radicals such as the imidazole, guanidine, and oxyphenyl groups. The change in the values of these latter groups in the peptide molecule over that in the amino acids is usually quite small. On the other hand, the alteration in the dissociation values of amino and carboxyl groups is quite large and is the resultant both of the influence of the peptide linkage and the nature of the substituted group or groups in proximity.

The presence of basic radicals in the molecule often results in a shift of the isoelectric point toward a lower acidity. For example, peptides like phenylalanylarginine, histidylglycine, and histidyl-histidine, containing the guanidine or imidazole groups, possess isoelectric points more acid than those of the free arginine or histidine molecules (7). This shift is reflected in the simultaneous increased acid strength of the carboxyl group and decrease in strength of the amino group which become clear on comparing

<sup>7</sup> While the validity of using the modified Gibbs-Helmholtz equation applied to the data presented may be questionable, owing to the numerous simplifying assumptions made in the calculations of  $(\Delta F^\circ)'$  and  $(\Delta H)'$ , it is believed that it is capable of an approximate description of the phenomena presented.

these values in this type of complex peptide with those of glycylglycine (Table I). The presence of the guanidine nucleus in arginine and that of the imidazole ring in histidine result in this same type of acid shift over the values characteristic for the monoamino-monocarboxylic acids. Thus glycine possesses a  $pK'$  value for the carboxyl group of 2.33 (1), arginine, however, one of 2.01 (4), and histidine 1.82 (4). The carboxyl dissociation value in glycylglycine is 3.12, that of phenylalanylarginine is 2.66, and of histidylglycine 2.40.

The intensified acidity of the carboxyl group due to basic substituents is, however, best illustrated by lysyllysine. This substance, containing three amino groups and one carboxyl group, may be considered as differing from lysylglutamic acid in that the former possesses an amino in place of a carboxyl group. The effect on the dissociation of the carboxyl group in lysyllysine due to the presence of this third amino group is very marked. The  $pK'$  value for the carboxyl group in lysylglutamic acid is 2.95; in lysyllysine it is 1.95. The corresponding dissociation value for the same group in lysine is 2.18. The customary weakening effect of the peptide linkage on the carboxyl group is more than counteracted by the presence of several amino groups and this phenomenal shift may be compared with the dissociation value found by the author (12) of 1.33 for the carboxyl group in  $\alpha, \beta$ -diaminopropionic acid. In the latter substance there is likewise achieved a great concentration of amino groups within a rather small molecular area. The peptide, histidylhistidine, which like lysyllysine also contains three basic groups and one carboxyl group, yields a low  $pK'$  value for the latter group of 2.25 (7).

Another type of substituent whose introduction into the peptide molecule results in a shift toward more acid values is the weakly acid hydroxyphenyl group. In tyrosine it leads to an increase in acid dissociation and a decrease in basic dissociation as compared with glycine (1). In tyrosyltyrosine and glycyltyrosine (8) it causes a slight shift toward a more acid isoelectric point than that of tyrosine. In tyrosylarginine it shifts the isoelectric point from 9.99 in phenylalanylarginine to a value of 8.38. The value of  $pI$  in aspartyltyrosine is slightly more acid than that of aspartylaspartic acid (7).

Lysylglutamic acid is the first peptide prepared of a diamino-

monocarboxylic acid and a monoaminodicarboxylic acid. Its properties should not be very different from those of glycylglycine and in point of fact its isoelectric point of 6.10 at 25° is only slightly higher than that of glycylglycine, namely 5.65, at the same temperature. Furthermore, the temperature coefficient of the isoelectric point of both compounds between 0–25° is 0.35 unit (Table I).

While amino and carboxyl groups are usually considerably weakened by the interposition of the peptide linkage, the shifts in the values of the substituted groups described above are small in comparison, and their direction is determined by the nature of the groups in proximity. It had been previously suggested that in histidylhistidine (7) the imidazole ring next to the amino group would be shifted to a more acid value than in histidine. Whereas in this peptide containing two imidazole rings it was difficult to assign a value to either ring, the results obtained now with histidylglycine confirm this assignment. In the latter peptide, the lone imidazole radical is located on the acyl amino acid and in  $\beta$  position to the amino group. Its  $pK'$  value at 25° is 5.80 as compared with 6.04 in histidine (4) and 5.60 in histidylhistidine (7). Similarly it had been suggested by the author (8) that the shift in the dissociation value of the hydroxyphenyl group would be determined by the nearness of a carboxyl or an amino group. In glycytyrosine, where the oxyphenyl ring is next to the carboxyl group, its dissociation is weakened and is shifted to a slightly more alkaline range. In tyrosyltyrosine, the value of the more acid  $pK'$  for the radical was therefore assigned to that nearer the amino group. That this assignment was correct is shown by the dissociation values found in tyrosylarginine and in aspartyltyrosine. In the former, where the oxyphenyl group is on the acyl amino acid, its value is 9.35; in the latter compound it is found on the distal amino acid and its value is 10.20. These values are respectively more acid and more alkaline than that  $pK'$  value of tyrosine, namely 10.07 (38). The dissociation values of the two rings in tyrosyltyrosine were 9.80 and 10.26, and the assignments previously made of these values, based on the structure of this compound, are confirmed by the results obtained in the present study.

In the case of the guanidine group, its shift toward a weaker value is slight; *i.e.*, from 12.48 in arginine to 12.40 in phenylalanyl-arginine and to 11.62 in tyrosylarginine (Table I). The strength

of this group will probably be less affected by neighboring substituents and will depend more on the number of peptide linkages in the chain. It is probable that arginine when in the acyl peptide position will yield a somewhat more alkaline value for the guanidine group than when found in the amino acid alone.

Glycylproline with its  $\text{—CON=}$  peptide linkage affords an interesting contrast with glycylglycine which possesses the  $\text{—CONH—}$  type of peptide binding. While the effect of both types of linkages is to weaken both amino and carboxyl groups as compared with the amino acid, the former linkage has a markedly less weakening influence than the latter. The dissociation values of the carboxyl groups are 2.84 and 3.12 in glycylproline and in glycylglycine respectively, with values of 8.62 and 8.17 for the respective amino groups. The isoelectric points of both compounds are practically identical, with 5.73 for glycylproline and 5.65 for glycylglycine. These results with glycylproline are comparable with those obtained by Levene, Simms, and Pfaltz (39) in the case of glycylsarcosine. This compound, with a type of peptide linkage similar to that of glycylproline, yielded  $\text{pK'}$  values for the carboxyl group of 2.83 and for the amino group of 8.54, with the isoelectric point at 5.68.

In Table II are gathered the data relating to the rates of deamination of lysine, lysyllysine, lysylglutamic acid, and lysylhistidine. Whereas lysine requires about 15 minutes at ordinary temperature for the quantitative removal of the  $\epsilon$ -amino group, it is found here that peptides containing lysine are remarkable in that their total amino nitrogen is removed in about half this time at the same temperature. In a personal communication from Professor M. Bergmann, the author was informed that similar results had been found in the former's laboratory. This rather rapid rate of deamination of the lysine peptides stands in contrast with the much longer time required to deaminate a protein like gelatin (40) or casein (14), and points to a difference in the surface constitution of the larger and smaller molecules. These differences in constitution most probably do not affect the ionization of the various groups, since these depend more upon the nature and character of contiguous groups as well as upon the distance between them. The  $\alpha$ -aminomonocarboxylic acids possess different constitutions and the rate of introducing substituents, as shown by Abderhalden

and collaborators (41-43) in the case of the conversion of the bromo derivatives of the isomeric valines and leucines into the amino acids and the betaines, will be clearly different in each case. However, their dissociation values are all approximately equal. It is highly probable that the chemical reactivity of the free groups involved in the protein or the amino acids bears little if any relation to their ionizing capacity, and the differences in the rates of

TABLE II  
*Amino Nitrogen Liberated at 22°*

Time min.	Lysine		Lysyllysine		Lysylglutamic acid		Lysylhistidine	
	per cent	$K^*$	per cent	$K^*$	per cent	$K^*$	per cent	$K^*$
1			73				63	
2	64		82		80		84	
3			87	0.29	85	0.27	90	0.33
4			90	0.25	91	0.26	92	0.28
5	84	0.17	92	0.22	94	0.25	96	0.28
6	87	0.15	95	0.21	95	0.22	98	0.28
6.5			96				99	
7	90	0.14	100		99		100	
8	91	0.13	100		100		100	
9			100		100		100	
10	95	0.13			100			
11	97	0.14			100			
12	98	0.14						
14	99							
15	100				100			
16	100							
18	100							

\*  $K$  = constant for the monomolecular reaction. Its average value for lysine is 0.14, for lysyllysine 0.24, for lysylglutamic acid 0.25, and for lysylhistidine 0.29. Dunn and Schmidt (14) report a value of  $K = 0.11$  at 23° for the  $\epsilon$ -amino group of lysine.

deamination of the  $\epsilon$ -amino group in lysine, lysine peptides, and the protein, must be sought in causes arising from differences in chemical structure other than those concerned with alterations in ionization.

I wish to thank Professor Carl L. A. Schmidt for his advice and suggestions during the course of the titration experiments herein recorded.

## SUMMARY

1. The apparent dissociation constants at 0° and at 25° have been determined and the free energy changes and heats of ionization have been calculated for phenylalanylarginine, tyrosylarginine, aspartyltyrosine, histidylglycine, phenylalanylglycine, glycylglycine, lysylglutamic acid, and lysyllysine.

2. It is shown that the heat of ionization of the carboxyl group is negligible, while that of the amino and guanidine groups is considerable, and of the order of 10,000 to 12,000 calories, whereas those heat values of the imidazole and oxyphenyl radicals possess intermediate magnitudes. Although the actual titration values of the free groups of the peptides are markedly different from those of the same groups when found in the amino acids, and may furthermore be influenced by the presence of other substituents, the values of the heats of ionization corresponding to these groups are apparently independent of the constitution of the molecule. In the case of peptides containing several free charges, the temperature coefficient thus permits some means of identification of the ionizing group.

3. The change in free energy due to ionization corresponding to the carboxyl group is slightly greater in the peptides than in the amino acids, and possesses an appreciable temperature coefficient. On the other hand, the free energy change of the amino groups in the peptides is considerably less than that of the same group in the amino acids and its value is practically independent of the temperature. The thermodynamic behavior of both groups and of the imidazole radical may be described in terms of the modified Gibbs-Helmholtz equation. The temperature coefficient of the free energy change of the carboxyl, hydroxyphenyl, and guanidine groups is always positive and of the same order of magnitude.

4. While in the transition of amino acids to peptides the amino and carboxyl groups are considerably weakened, this weakening may be modified by the presence of various groups such as the imidazole and oxyphenyl rings, and the guanidine nucleus. A concentration of basic groups as in lysyllysine may even more than overcome the weakening influence of the peptide linkage and cause the carboxyl group to dissociate at a reaction more acid than in lysine itself. The shifts in the values of these ionizing radicals



are small in comparison to that undergone by amino and carboxyl groups, and their direction is determined by the position of these groups in the molecule.

5. The titration constants at 25° have been determined for glycylproline and compared with those values for glycylglycine. These compounds differ in the nature of the peptide bond. It was found that the weakening influence of the peptide linkage of the former compound on amino and carboxyl groups is considerably less than that of the latter. However, the isoelectric points of both substances are practically identical.

6. The rates of deamination by means of nitrous acid of lysine, lysyllysine, lysylglutamic acid, and lysylhistidine have been determined. Whereas lysine requires approximately 15 minutes for complete deamination, the lysylpeptides all require about half this time.

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## THE LIPID CONTENT OF THE WHITE BLOOD CELLS IN NORMAL YOUNG WOMEN

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During the course of a previous investigation (Boyd, 1931) it was noted that a moderate venisection rendered available sufficient white blood cells for lipid analysis by the micro-methods of Bloor and his associates. At that time the phospholipid content of leucocytes was determined in four dogs, revealing a relatively high percentage, 710 mg. per cent, of lecithin compounds. In view of the obvious importance of the white blood corpuscles in health and disease, a series of experiments was undertaken to establish the normal values for the lipid content of these cells in human blood. Inasmuch as further problems under investigation have to do with the variation of the leucocyte lipid content in menstruation, pregnancy, and diseases of women, the normal values herein reported are those of young, healthy, non-menstruating women.

While there have been numerous histological studies made on the white blood cells, little is known concerning their physiological chemistry. Willstätter and Rhodewald (1931, 1932) have determined the presence of lyomaltase and desmomaltase in the white cells of horse blood. Similarly Stern (1932) has found catalase present in white cells. Such studies suggest that the enzyme content of leucocytes is not unlike that of the other active tissues of the body. Of more particular interest to the question of lipid metabolism in the white cells are the findings of Hirsch (1928) that lecithin given intravenously stimulates a leucocytosis with a relative lymphocytosis, whereas cholesterol under the same conditions produces a polymorphonuclear leucocytosis. Considered in the light of the conclusions reached by Garrey and Butler (1932) that ingestion of proteins or carbohydrates has no

effect on the basal white cell count, it is suggested that the so called "digestive leucocytosis" is a reaction specific to ingested fats.

### *Methods*

Eight normal young women in the 20 and 30 year age groups acted as subjects for this investigation. According to the menstrual history, each subject was within the middle 2 weeks between menstrual periods, at least 1 week and not over 2 weeks from the previous period which was, in each case, of normal flow and duration. These facts were carefully ascertained since it has been shown by Okey and Stewart (1932-33) that menstruation affects the level of blood (whole) lipids. Their height and weight, which was within average limits, having been determined, each subject was put on a normal balanced diet and kept in bed for at least 24 hours previous to drawing blood for analysis. A brief but complete physical examination, including blood Wassermann, cell count and hematocrit, temperature, pulse and respiration, and urinalysis, was made on each woman to assure normal health and rule out particularly infection, tumor or malignant growths, uterine bleeding, etc., which are known to affect the lipid content of whole blood, serum, or plasma (Bloor, 1932). Since in each case the data obtained indicated the subject was in all respects normal, such protocols have not been included here.

No food was allowed for 16 hours preceding the test. A 50 to 60 cc. sample of blood was withdrawn from the arm veins at 8.30 to 9.00 a.m. following the fast. This was shaken in a flask previously rinsed with saturated sodium citrate and immediately centrifuged for  $\frac{1}{2}$  to  $\frac{3}{4}$  of an hour. The greater portion of the plasma was then drawn off with a pipette, the remaining amount being absorbed with thin strips of dry, fat-free filter paper. An adherent white cell layer was thus left on the surface of the red cells which could be readily removed by a pair of clean forceps. This was transferred to a weighed flask and its weight determined. Usually about 0.5 gm. of white cells was obtained.

The leucocyte fraction was ground in a mortar with sand and then extracted with about 75 cc. of redistilled alcohol-ether in the proportion 3:1, according to the method of Bloor (1928, a). The solvent with the triturated cells was then heated to a gentle boil

on the steam bath for about 5 minutes and let stand for an hour. The resulting extract was filtered through an alcohol-extracted filter paper into a 100 cc. volumetric flask and made up to volume after washing the precipitate with several portions of alcohol-ether. By this procedure a 1:150 to 1:250 diluted extract of the original cells was obtained.

Suitable aliquots of the extract were then pipetted off for analysis of the various lipids. It was necessary to determine on preliminary samples the quantity of extract required to give a sufficient amount of lipid for each particular lipid determination. These surveys indicated that about 20 cc. of the alcohol-ether extract were sufficient for the total fatty acids and total cholesterol estimations while 40 to 50 cc. were used for the joint estimation of phospholipid and free cholesterol, the latter being determined upon the acetone-petroleum ether mother liquor and washings of the former as described below.

On each extract total fatty acids, free and total cholesterol, and phospholipid were directly determined and from these values the total lipid, neutral fat, combined cholesterol, and distribution of the fatty acids calculated. Since iodine number estimations require relatively large amounts of lipid, it was found impossible to include these as part of the routine analysis. However, in order to have some idea of the unsaturation of the fatty acids of leucocytes, a few iodine numbers were run on separate samples, using, for this purpose, the entire 100 cc. of alcohol-ether extract. The details of this composite lipid analysis have been reported elsewhere (Boyd, 1933), so that merely a brief description of the principles and procedures need be indicated here.

The analysis was based on the combined use of a series of micro-methods for lipid determinations which have been evolved chiefly by Bloor and his associates (Bloor, 1928, *a*, 1929; Boyd, 1931, 1933; Dam, 1930; Okey, 1930; Page, Pasternack, and Burt, 1930; Yasuda, 1931, 1931-32) in which the particular lipid in question is isolated by selective solubility or precipitation and then completely oxidized with chromic acid. The lipid molecule, being of high molecular weight, requires, under these circumstances, a titratable amount of the oxidizing agent varying from the equivalent of 3.00 cc. of 0.1 N potassium dichromate per mg. (phospholipid) to 10.62 cc. (cholesterol digitonide). It may thus be seen that

amounts of lipid as low as 0.5 to 1 mg. may be quite accurately estimated. The results obtained by this oxidative procedure approximate those derived by the use of methods based on entirely different principles such as acid titration (Man and Gildea, 1932-33), nephelometry, colorimetry, etc. (see review of micromethods by Bloor, 1932). However, for the purpose of a differential analysis of the lipid content of a single tissue, there is a distinct practical as well as theoretical advantage in the use of a series of methods all based on the same principle. The oxidative principle is the only one so far evolved which may be applied to the micro-determination of most of the common lipids. Recognizing this, the author has recently described in detail a procedure for the composite analysis of blood plasma lipids based on oxidative methods (Boyd, 1933). The same procedure has been applied to the analysis of leucocyte lipids reported below, and may be briefly outlined as follows:

A suitable aliquot of the alcohol-ether extract was saponified and extracted with petroleum ether, thereby isolating the total fatty acids and total cholesterol which were oxidized. On a second petroleum ether aliquot the total cholesterol was determined by precipitation as the digitonide and oxidized; by subtraction, the percentage of total fatty acid may thus be determined. From a further portion of the alcohol-ether extract the phospholipids were prepared in petroleum ether solution and precipitated with acetone and magnesium chloride, redissolved in moist ether, evaporated, and oxidized. The acetone mother liquor from which the phospholipid was precipitated contains free cholesterol which may be determined by digitonin precipitation and subsequent oxidation. The difference of the per cent of free cholesterol from the per cent of total cholesterol gives the per cent of combined cholesterol from which may be computed the cholesterol ester (as stearate or oleate) and the cholesterol ester fatty acid (0.67 times the combined cholesterol). Phospholipid fatty acids may be estimated as two-thirds the total phospholipid.

The sum of the cholesterol ester fatty acids plus the phospholipid fatty acids subtracted from the total fatty acids indicates the fatty acids combined with glycerol as neutral fat. Since fatty acids compose, on the average, 95 per cent of neutral fat, the latter may be readily deduced from the former percentage. Fi-

nally, the total lipid present may be found from the sum of the neutral fat plus phospholipid plus total cholesterol plus cholesterol ester fatty acids. It may thus be seen that a composite view of the lipids present in a tissue may be obtained by this procedure.

### *Results*

In Table I are listed the results of a series of analyses of the lipid distribution in the white blood cells of eight normal young women as determined by the preceding methods. Due to the small amount of tissue extract available, it was possible to make only single estimations for each lipid. However, the range of experimental error and the reliability of the methods were determined beforehand by duplicate and triplicate determinations on preliminary extractions. It has been previously shown by the author (Boyd, 1931) that there is a 2 to 5 per cent standard deviation in the values obtained by the phospholipid procedure and later it was shown (Boyd, 1933) that a similar low range of experimental error existed in the other lipid micromethods. While these figures were obtained on blood plasma extracts, preliminary trials indicated like values prevailed in white blood cell extracts.

From the results of the several analyses the mean value for each lipid has been calculated and the standard deviation found from the formula  $\sigma = \sqrt{\Sigma (x)^2/n}$  where  $x$  represents the deviation of each value from the mean;  $n$  is the number of determinations;  $\sigma$ , the standard deviation; and  $\Sigma$ , a summation symbol (Dunn, 1929). It may be seen from Table I that the total lipid content of the white cells varies from 1 to 3 per cent with a mean value of 1710 mg. per cent (mg. per 100 gm.) and a standard deviation of 734 or 42.9 per cent of the mean. This lipid is distributed, in round numbers, as follows: phospholipid, 47 per cent; neutral fat, 31 per cent; free cholesterol, 11 per cent; and cholesterol ester, 11 per cent (calculated as the sum of the combined cholesterol and the cholesterol ester fatty acids).

Total fatty acids were present to the concentration of 1103 mg. per cent constituting 64.6 per cent of the total lipid. Of these, phospholipid fatty acids constituted 48 per cent having a value of 534 mg. per cent; neutral fat fatty acids comprised 46 per cent of the total fatty acids or 508 mg. per cent; and cholesterol ester fatty acids represented a comparatively small percentage of the total



TABLE I  
*Composition of Lipid Present in White Blood Cells of Normal Young Women*  
 The lipid values are measured in mg per 100 gm of white blood cells

Subject	Age	Height	Weight	Total lipid	Composition of total lipid								
					Neutral fat	Fatty acids			Cholesterol			Phos- pholipid	
						Total	Phos- pholipid	Choles- terol ester	Neutral fat	Total	Free		Com bined
J F	20	156	53.6	1343	346	812	304	179	329	361	71.7	269.3	457
D E	22	160	45	1172	239	730	498	5	227	181	173	7	747
M F	22	156	49	1963	583	1271	660	58	553	332	245	87	990
M H	28	161.5	65	1029	74.7	474	381	123	70	259	75.8	183.2	572
E J	28	155	48	1330	216	775	428	142	205	329	117	212	643
E A	34	151	71.6	2116	644	1390	778	0	612	304	304	0	1168
C B	36	167.5	74	1312	308	814	447	75	292	257	145.5	111.5	672
I M	38	153	52.6	3413	1874	2555	776	0	1779	375	420	0	1164
Mean	28.5	157.5	57.4	1710	536	1103	534	73	508	300	194	110	802
Standard deviation	6.4	14	10.5	734	536	614	170	65	508	60	110	97	255

fatty acids, 6 per cent or 73 mg. per cent, of the white cells. Compared with human blood plasma, the white cells contain approximately 3 to 4 times as much total fatty acid (see Bloor, 1921; Page, Pasternack, and Burt, 1930; and Boyd, 1933). Further comparison with human plasma reveals that in white cells a much higher percentage of the total fatty acid is present as phospholipid fatty acid, and conversely, a lower percentage as cholesterol ester fatty acids.

The mean determined value for total cholesterol was 300 mg. per cent or about 17.5 per cent of the total lipids. The total cholesterol was divided into 65 per cent free cholesterol and 35 per cent combined cholesterol, the figures in terms of mg. per 100 gm. of cells being 194 mg. per cent and 110 mg. per cent respectively. In contrast to human plasma (Boyd, 1933; Bloor, 1921; Okey and Stewart, 1932-33) the leucocytes contain 50 to 100 per cent more total cholesterol, about the same mean percentage of combined cholesterol, and about 4 times as much free cholesterol. As a result cholesterol esters compose a much higher percentage of the total cholesterol in plasma than in leucocytes.

Of especial interest, however, was the relatively high phospholipid content of the white cells, 802 mg. per cent or almost one-half of the total lipid. This finding corroborates the high phospholipid value, 710 mg. per cent previously found by Boyd in the white blood cells of dogs (1931). Such phospholipid values are 4 to 5 times greater than those of normal human plasma and double that of the red cells (Bloor, 1921).

We may now turn to a consideration of the standard deviations as listed in Table I. It will be readily seen that for all lipids the normal values have a considerable range. The greatest variation is in the neutral fat where there is a 100 per cent standard deviation and actual experimental values varied from 74.7 mg. per cent to 1874 mg. per cent. The latter figure was obtained in a woman of 38 years who had led a life of rather hard work, had given birth to nine children, and looked rather older than her years indicated. In contrast, the other seven subjects of this series were either nulliparous or had one or two children.

Cholesterol ester cholesterol also showed a marked variation, values from 0 to 269.3 mg. per cent being recorded with a 90 per cent standard variation. The remaining lipids showed a much

smaller degree of variation, free cholesterol being next highest with a 56.7 per cent standard deviation followed closely by a 55.7 per cent standard deviation for the total fatty acids. Total lipids had a 42.9 per cent  $\delta$  and phospholipids a 31.8 per cent  $\delta$ ; total cholesterol was the least variable lipid of all, giving a 20 per cent  $\delta$ .

As stated above, iodine number determinations were not made routinely on each alcohol-ether extract due to insufficiency of extract. However, in view of the interest in the phospholipid content of the white cells, a further extract was used entirely for estimation of the iodine number of the phospholipid fatty acids. The value obtained, with the Yasuda (1931-32) technique was 61.2. This figure indicates that the phospholipid fatty acids of the white cells are relatively more saturated than those of plasma of the human (Boyd, 1933) but further determinations are necessary before definite conclusions can be drawn.

#### DISCUSSION

The results of the above lipid analysis of human white blood corpuscles offer some interesting considerations and comparisons with the lipid content of human blood plasma and the body tissues generally. They possess a total lipid content approximately 4 times as great as normal blood plasma with a marked variation in the percentage composition of the component lipids from the latter. In some respects, however, the lipid analysis of the white cells resembles that of plasma, whereas in other respects it more closely approximates that of the body tissues. If we use as a basis for comparison the lipid analysis of the skeletal muscles, heart muscle, liver, kidney, pancreas, brain, etc., of the cow as determined by Bloor (1926, 1927, 1928, *b*), it may be seen that white blood cells resemble these body tissues in their high total lipid and relatively large proportion of phospholipid. The similarity is more pronounced toward the tissues of high cellular and nuclear content as liver and kidney than toward the muscles.

The leucocyte lipids differ from those of the body tissues in the relatively large proportion of cholesterol esters found in the former and in this respect the white cells tend toward the composition of blood plasma lipids. The range of cholesterol esters content of the white cells, however, is quite extensive, extending from 0 to 75 per cent of the total cholesterol as seen in Table II. In plasma

the cholesterol esters constitute 60 to 70 per cent of the total cholesterol with little tendency to variation in normal subjects (Boyd, 1933), whereas in body tissues esters rarely exceed 10 per cent of the total cholesterol. Bloor, Okey, and Corner (1930), in a study of the corpus luteum of the sow, found that a high percentage of cholesterol ester characterized the degenerating gland.

On the other hand, a high percentage of phospholipid appears to indicate increased functional activity of a tissue (Bloor, 1926, 1927, 1928, b). Bloor, Okey, and Corner (1930), in the investigation noted above, found a marked increase in phospholipid with activity of the sow corpus luteum. From their work it may thus be concluded that active cells contain a high percentage of phospholipid

TABLE II  
*Ratios between Lipids of White Blood Cells in Normal Young Women*

Subject	M H	D E	E A	E J	C B.	M F	J F.	I M.
Total fatty acid								
Phospholipid	0 83	0 98	1 19	1 21	1 21	1 28	1.78	2.19
Phospholipid								
Cholesterol	7 53	4 32	3 84	5 50	4 62	4 03	6 38	2 65
Ester cholesterol								
Total cholesterol	0 708	0 039	0 0	0 645	0 434	0 262	0 746	0 0

and little or no cholesterol ester, while inactive or degenerating cells possess a low content of phospholipid and an increasingly high amount of cholesterol esters. It is therefore of interest to note that those white cells which contained the highest percentage of cholesterol ester cholesterol (see Table I) had the lowest percentage of phospholipid, suggesting relatively inert or inactive cells. Conversely, when the cholesterol ester cholesterol of the white cells was found low, the phospholipid values were highest, presumably due to increased activity on the part of the cells. On the face of this evidence it would appear that these cells were not all in the same stage of functional activity, although judged by the usual standards they were apparently all the same. Further investigation is being carried on in this connection.

Finally, there is left a consideration of the variation noted in

the lipid values from one white cell extract to another. Whereas it is well known that the absolute values for the lipid content of a tissue may vary considerably, certain quantitative proportions between various lipids have, in general, been found to exhibit a degree of constancy. In Table II have been recorded values for two of the commonest such ratios, phospholipid to cholesterol and total fatty acid to phospholipid. The additional data in Table II regarding the relative amounts of free and combined cholesterol have been included for the considerations noted above. It may be seen that the phospholipid to cholesterol ratio varied from 2.65 to 7.53, and the mean value may be calculated to be 4.86 with a standard deviation of 1.45 or 30 per cent  $\sigma$ . As previously computed there is a 31.8 per cent  $\sigma$  for phospholipid values and a 56.7 per cent  $\sigma$  for free cholesterol. It may thus be concluded that the phospholipid to cholesterol ratio is more constant than the figures for the separate lipids of the ratio and that whereas the absolute values of phospholipid and cholesterol may vary markedly from cell to cell, there is a tendency to maintain constant the relative proportion of one lipid to the other in any given white cell extract. Similarly the per cent  $\sigma$  for the ratio, total fatty acid to phospholipid, is 27.5 per cent as against a per cent  $\sigma$  for the two component lipids of 58.7 and 31.8 respectively. Here again is indicated a constancy in the ratio as compared with the variation in the absolute values of the separate lipids.

#### SUMMARY

From an analysis by oxidative micromethods of the lipids present in the white blood cells of eight normal young women, it has been found that the total lipid content is 1 to 3 per cent. This is composed, in round numbers, of 47 per cent phospholipid, 31 per cent neutral fat, 11 per cent free cholesterol, and 11 per cent cholesterol ester. The total fatty acids derived from these lipids compose approximately two-thirds of the total lipid. From the experimentally determined and calculated values a differential analysis of the lipids in the white blood cells has been tabulated and discussed. It has been shown that while there is a considerable variation in the absolute values of the various lipids, some greater than others, there is a tendency to maintain a constant proportion of one lipid to the other.

Comparison of these data with published values for the other tissues of the body places the white blood cells intermediate between the body tissues and blood plasma with respect to their lipid content. The relation of functional activity to lipid composition of the leucocytes has been discussed.

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## THE ABSORPTION OF CERTAIN SULFUR COMPOUNDS FROM INTESTINAL LOOPS OF DOGS\*

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Although the rate of absorption of amino acids from the small intestine has been frequently studied (1-5), the substances in question have usually been introduced either by mouth or by stomach tube. The use of isolated intestinal loops, although a less common procedure, has nevertheless certain advantages in that the influence of such factors as variations in gastric motility, the effect of gastric acidity on the pH of the intestinal contents, and decomposition produced by bacteria from the lower intestine is avoided. Preliminary to some metabolic studies of certain sulfur compounds, the relative rates of absorption of these compounds from jejunal loops of dogs were measured. Thirty loops, prepared as described by Johnston (6), were used and the animals were kept in healthy condition on a normal diet for many months. There was no evidence of any abnormal condition in the loop and absorption studies, both shortly following the operation and months afterward, showed no indication that abnormal changes had occurred in the isolated segment of intestine.

The technique consisted merely in quantitatively introducing weighed samples into the loop and removing what material remained at the end of the experimental period. The opening of the loop was sealed by means of a double rubber balloon valve provided with a catheter which extended the full length of the loop. Through this tube, the substance was introduced and removed by

\* An abstract of this paper was presented at the meetings of the Federation of American Societies for Experimental Biology, held in Cincinnati, April, 1933.



means of a 50 cc. syringe. Both *l*-cystine and *dl*-cystine were administered as a water suspension in very finely ground form. Although far from being ground to colloidal dimensions, this material was sufficiently fine to permit quantitative introduction through the syringe. A total of 50 cc. of water was used (in small portions) to wash the sample completely into the loop. However, the quantity of water introduced was varied at different times from about 40 to 80 cc. and still wider variations were encountered in the volume of fluid recovered at the end of the experiment when secretion into the intestine had raised the volume of the contents to 80 to 200 cc. In all cases a larger volume was removed than was introduced. Similarly, at the end of the experiment the loop was washed repeatedly to insure quantitative recoveries. Immediate recoveries on each of the substances investigated showed that with proper technique it was possible to recover from 95 to 100 per cent. The greatest problem in quantitative recovery was presented by the cystine samples. For these a wash with dilute alkali was found necessary since the suspensions quickly settled out in the intestine and could not be recovered quantitatively by washing with water. However, dilute sodium hydroxide (0.001 to 0.01 N) used after several preliminary washings with water and introduced in quantity sufficient to dilate the loop moderately (about 200 cc.) followed by further washings with water gave recoveries of nearly 100 per cent. There was no evidence that the alkali used produced any irritative effect on the loop. During the experiment the dogs were allowed normal animal house freedom with the outlet of the loop and the balloon firmly bandaged.

The experimental period was, in most cases, 4 hours. This length of time was selected chiefly on grounds of convenience and was found to be well suited to the rate of absorption of all compounds studied except cysteic acid (see below), the extremely rapid absorption of which required shorter periods. The sample used was uniformly of 0.5 gm. in the case of cystine and, for the other substances, was such as to contain an equivalent amount of sulfur: cysteine, 0.504 gm., cysteic acid, 0.703 gm., and sodium sulfate, 0.591 gm.

The *dl*-cystine was prepared by racemization of *l*-cystine in boiling hydrochloric acid. The cysteine was prepared both by electrolysis and by tin reduction. It was preserved under nitro-

gen in strong HCl solution. Just before introduction into the loop, the required volume was neutralized to pH 7 with NaOH solution. The free cysteine was therefore introduced along with some sodium chloride. Cysteic acid was introduced as the monosodium salt which is practically neutral in reaction.

The contents and washings from the intestinal loops were at once acidified with HCl to prevent any bacterial decomposition and (in case of cystine) to neutralize the slight amount of alkali, and made up to volume in a 1 liter flask. The solution was then filtered and aliquots were used for analysis. Cystine was determined by the Folin method and also by the Benedict determination of total sulfur, weighed as  $\text{BaSO}_4$ , the results between the two agreeing closely. For the other substances the Benedict sulfur method was used. It was also possible to obtain consistent figures by one direct sulfur determination on the unfiltered solution by making a previously determined blank correction for the sulfur in the mucus and the secretion washed out of the loop. The quantity of sulfur obtained in these blanks was remarkably constant (0.034, 0.029, 0.038, and 0.035 gm. of  $\text{BaSO}_4$ ).

Table I shows the results obtained on two dogs. The figures are expressed in terms of percentage recovery of the substance. Although the results are fairly consistent for any one animal, the variations between animals in the case of cystine are larger. The two animals listed in Table I, however, represent the maximum variation encountered. The slightly increased speed of absorption of *dl*-cystine over *l*-cystine confirms the results reported by Lawrie (5) with rats. This is no doubt, as Lawrie suggested, the result of the higher isoelectric solubility of *dl*-cystine, but the difference, as was the case with his experiments, is small.

Nitroprusside tests were made on all material recovered from the loop. 4 hours after administering cysteine, the nitroprusside reaction was usually, although not always, negative, thus indicating that considerable reoxidation had taken place. At the pH of intestinal contents this would be expected. This naturally implies a higher rate of absorption for pure cysteine than is indicated by the figures in Table I.

Nitroprusside tests on the material recovered after cystine administration were uniformly negative. Although these loops were not completely sterile, there was no evidence of the presence

of sufficient bacteria to produce any hydrogen sulfide or sulfhydryl derivative and there appears to be no evidence of any reduction to cysteine before absorption. The lack of formation of any compound which would respond to the nitroprusside test was further demonstrated by an experiment in which 5 gm. of *l*-cystine were administered by stomach tube to a dog with a jejunostomy.

The animal was prepared by bringing a piece of intestine (about 22 cm. below Treitz's ligament) out through the abdominal wall.

TABLE I

*Percentage Recoveries of Sulfur Compounds from Intestinal Loops of Dogs*

Time, 4 hours; sample used, 0.500 gm. of cystine or its sulfur equivalent.  
No cysteic acid was recovered from either dog

	<i>l</i> -Cystine	<i>dl</i> -Cystine	Cysteine	Na <sub>2</sub> SO <sub>4</sub>
Dog 941, 18.3 kilos	68.0	64.2	45.7	82.3
	79.2	76.0	31.0	67.7
	70.0	58.0	22.5	72.1
	78.4	60.0	15.8	69.7
			14.9	70.4
Averages	74.0	64.5	26.0	72.5
Dog 36, 27.9 kilos	29.9		36.8	60.0
	30.2		26.0	64.5
	37.0		23.5	60.4
	33.3		11.4	57.7
	30.0		24.6	
	35.0			
	29.1			
Averages.	32.1		24.5	60.6

The abdominal wall was closed about the intestine, the continuity of the gut severed, and the two ends of the intestine connected by means of a T-tube the free end of which was closed. After fasting the animal for 2 days, a rubber balloon was placed on the free end of the tube. The intestinal fluid which was collected showed no evidence of gross blood. 5 gm. of cystine and a small amount of norit were given by stomach tube and washed down with 100 cc. of 0.1 N HCl. Carbon appeared in the drainage within 45 minutes and, with the arm of the T-tube leading into the distal

segment of gut clamped, the drainage was continued. The material collected in 5 hours contained 75 per cent of the cystine originally administered but showed no cysteine or hydrogen sulfide, either by nitroprusside test, iodine titration, or by the colorimetric Folin method with omission of sulfite. All analytical evidence indicated that no other sulfur compound than the unabsorbed cystine was obtained from the intestine.

Experiments with fresh hashed jejunum to which cystine has been added have uniformly demonstrated that hydrogen sulfide and cysteine are produced freely if the mixture is incubated for several hours at 38° without protection from the action of intestinal bacteria but that the formation of these substances is completely inhibited in the presence of chloroform. Under the latter conditions after 3 days incubation it was still possible to recover quantitatively the cystine as such and the solution gave no evidence of any sulfur reduction products.

It appears, therefore, that, barring the action of intestinal bacteria, there is no evidence to support the assumption of any chemical change in which the cystine is altered before absorption. There is a possibility of the cystine undergoing the Vickery reaction (7) as a preliminary metabolic step and in experiments with loops the very rapid rate of absorption of both cysteine and cysteic acid still leaves open the question of such a mechanism. However, the experiments *in vitro* with hashed intestine referred to above not only showed no cysteine formation when bacterial action was excluded but have in all cases given negative results on our attempts to isolate cysteic acid from the mixture.

The extremely rapid absorption of cysteic acid is noteworthy. In a 2 hour period for this substance, Dog 941 gave a series of recoveries varying from 16 to 26 per cent. Similar figures were obtained from Dog 36. It was also noted that on successive 2 hour experiments with cysteic acid on both dogs, the experiments immediately following each other, the rate of absorption of cysteic acid decreased somewhat (the recovery percentages rose). Thus, in one such case five successive periods raised the recoveries consistently from 16 per cent at the start to 24 per cent at the end. Similar figures were obtained in other such cases. The rise, while not great, was regular and consistent.

The rapid absorption of cysteic acid appears to be without effect

on the slower absorption of cystine when both are carried out together. Simultaneous introduction of 0.5 gm. of cystine and 0.703 gm. of cysteic acid for 4 hour periods gave, as usual, zero recoveries for cysteic acid and cystine recoveries no different than if the cystine had been used alone.

The very similar figures obtained for cystine and sodium sulfate are of course entirely accidental. The slowness of cystine absorption is no doubt partly the result of its insolubility whereas the low rate of absorption of sulfates from the small intestine has been commented on by other investigators (8).

Preliminary fasting of the animal did not influence the rate of absorption. Variations in the volume of water introduced with the sample had no detectable effect, nor did variations in the amount of fluid secreted into the intestine during the course of the experiment.

#### SUMMARY

The relative rates of absorption from isolated jejunal loops of the dog of the following substances have been measured: *l*-cystine, *dl*-cystine, cysteine, cysteic acid, and sodium sulfate. Of these by far the most rapid absorption was observed with cysteic acid. *l*-Cystine and sodium sulfate were absorbed least rapidly.

A slightly higher speed of absorption of *dl*-cystine as compared with *l*-cystine was observed.

No evidence was obtained of any reduction of cystine during 4 hour periods in the jejunal loop.

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## FACTORS INFLUENCING THE MEASUREMENT OF THE PHOSPHATASE ACTIVITY OF TISSUE EXTRACTS

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Within recent years a number of reports have appeared indicating that a relationship exists between various diseases in man and animals and quantitative changes in the phosphatase content of the tissues. According to Robison and Soames (1) and to Demuth (2) the phosphatase content of the bone is high in rachitic animals. Heymann (3) has reported a reduction in the phosphatase content of the upper intestinal wall and the kidney in rachitic rats. Kay (4) reports high plasma phosphatase values in a number of bone diseases (rickets, fragilitas ossium, osteitis fibrosa, osteomalacia, osteitis deformans) and in hyperthyroidism.

The validity of comparing the enzyme content of tissue extracts depends upon the extent to which a direct proportionality between enzyme activity and concentration exists; *i.e.*, upon whether an observed  $n$ -fold activity actually represents an  $n$ -fold concentration of enzyme. The purpose of this investigation was to determine to what extent this held true for the phosphatase activity of tissue extracts.

The influence of such known factors as pH and magnesium concentration and of hitherto unconsidered factors such as the concentration of protein degradation products was evaluated. Two methods of expressing enzyme activity were compared with respect to their suitability for representing the amount of phosphatase in tissue extracts.

*Preparation of Crude Tissue Extracts*—The method used was essentially that described by Kay (5). Rat tissues were ground thoroughly in a mortar with sand. 20 volumes of distilled water

and 1 volume of toluene were added, the mixture was extracted at room temperature for 48 hours, filtered, and kept in the ice box. The small intestines were washed free of contents with physiological saline solution before extraction; the bone extracts were prepared from the vertebrae and the leg bones, and the kidney extracts from the whole kidneys.

The cattle tissues were obtained fresh. The intestines were washed with saline. The bone of the calf was found more suitable than that of the adult animal because of its greater activity; the fused tibia and fibula were used. The bones were cut or broken into small pieces, ground in a mill with sand, and extracted with 10 volumes of water and 0.5 volume of toluene.

*Technique of Hydrolysis*—The hydrolyzing mixtures were prepared as follows: To 1 cc. of 3.12 per cent crystalline sodium glycerophosphate and 0.4 cc. of 10 per cent sodium diethylbarbiturate, a small amount of sodium hydroxide or hydrochloric acid was added to adjust the pH, and sufficient water so that when the tissue extract was added the total volume amounted to 8 cc. The reaction was carried out in an electrically controlled water bath at  $24.00 \pm 0.05^\circ$ . At intervals 1 cc. was withdrawn from the hydrolyzing mixture and added to 4 cc. of 10 per cent (in later experiments 5 per cent) trichloroacetic acid. Inorganic phosphate was determined by the method of Fiske and Subbarow (6). The inorganic phosphate content at zero time was calculated from the content of the enzyme preparation.

*Substrate*—The sodium glycerophosphate used in these studies was prepared from a single batch of the salt obtained from the Eastman Kodak Company. According to Kay and Lee (7) the Eastman Kodak preparation consists almost completely of  $\beta$ -glycerophosphate. The concentration of the glycerophosphate in the hydrolyzing mixtures was 0.0127 M which on complete hydrolysis would yield 0.394 mg. of phosphorus as inorganic phosphate per cc. of hydrolyzing mixture.

*Buffer*—Sodium diethylbarbiturate, in a concentration of 0.5 per cent of the hydrolyzing mixture (0.0244 M) was used in all instances. In this concentration it is without effect on phosphatase activity (Table I). At a concentration of approximately 0.06 M, sodium diethylbarbiturate is also without effect whereas glycine, which has been used as a buffer at about this concentra-

tion by other workers (3, 5), retards the activity about 30 per cent. The influence of amino acids on phosphatase activity will be discussed more fully below.

*pH*—Several studies (1, 4, 5) indicate that the optimal pH for glycerophosphatase activity of tissue extracts is in the vicinity of 9.0. (The phosphatase of the red blood cell appears to be an exception with an optimal pH below 7.0 (8).) Since the zone is a narrow one (pH 8.8 to 9.1) and the slopes on both sides of the opti-

TABLE I

*Effect of Sodium Diethylbarbiturate and Glycine Buffers on Phosphatase Activity*

Buffer	Concentration of buffer per liter of hydrolysing mixture		$Q \times 10^{-3}$ Reaction velocity	Retardation per cent
	<i>mols</i> $\times 10^{-2}$	<i>gm.</i>		
Sodium diethyl- barbiturate	0 00	0 00	2 50	0 0
	0 61	1 25	2 55	-2 0
	2 44	5 00	2 50	0 0
	6 10	12 5	2 42	3.2
	12 2	25 0	2 01	19 5
Glycine	0 00	0.00	1 43	0 0
	1 25	0 94	1 50	-4 9
	6 25	4 70	1 02	28 7
	0 00	0 00	5 22	0 0
	1 25	0 94	5 07	2 9
	6 25	4 70	3 37	35 3

Substrate 0.0127 M glycerophosphate, temperature 24°; cattle bone phosphatase (Preparation CBD) in 37.5 per cent concentration with sodium diethylbarbiturate, 25 per cent in the first experiment with glycine, 75 per cent in the second glycine experiment.

imum steep, four or more hydrolyses, at pH values in the immediate vicinity of the optimum, have been run in all experiments recorded in this study. The maximum rates experimentally obtained have been used and all descriptions and calculations are, therefore, for phosphatase activity at optimal pH. The pH adjustments were made by adding small amounts of sodium hydroxide or hydrochloric acid, as previously described. In the study of the activity of tissue extracts, where the turbidity, protein, and salt content of the different extract concentrations are variable



factors, it is unwise to depend upon colorimetric determinations except as rough approximations.

*Method of Expressing Phosphatase Activity*—In the studies on the phosphatase activity of various tissue extracts in disease referred to above, the amount of phosphorus liberated as inorganic phosphate in a given time from various substances has been used as the measure of enzyme concentration. This measure has also been used in studies of the relation between enzyme concentration and reaction velocity (9-12).

In this study the reciprocal of the time in minutes ( $Q$ ) necessary to liberate 0.05 mg. of phosphorus as inorganic phosphate has been used to express enzyme activity. This method has been employed by Hudson (13) and by Nelson and Vosburgh (14) for invertase and by Northrop (15) for pepsin. It has the advantage over the first method in that the concentration and hence the retardant effect of the reactant products is kept constant as the enzyme concentration is varied.

The relation between varying amounts (6.25 to 75.0 per cent of hydrolyzing mixture) of active extracts of rat and cattle tissues and the observed phosphatase activity, as measured by the amount of phosphorus liberated in 30 minutes, was studied. Though the time used was considerably shorter than that employed by most observers, the activity, measured in this way, was considerably less than expected at the higher enzyme concentration for all tissues used, excepting cattle bone. That the linearity in the case of cattle bone is accidental and spurious will become apparent when the influence of accelerating substances in cattle bone extracts is considered below.

In Chart 1 the activities of varying amounts of tissue extracts are compared with the reciprocal of the time ( $Q$ ) necessary to liberate 0.05 mg. of phosphorus per cc. of hydrolyzing mixture as the measure of phosphatase activity. The time was determined by graphic interpolation from a time-change curve representing the early course of hydrolysis.

With rat bone extracts the relation is a linear one indicating a direct relationship between enzyme activity and tissue extract concentration varying from 6.25 to 75 per cent of the hydrolysis mixtures. With cattle intestine, rat intestine, and rat kidney extracts there is a relative retardation of activity at the higher

enzyme concentrations. With cattle bone extracts, on the other hand, there is a relative acceleration in the higher enzyme concentrations.

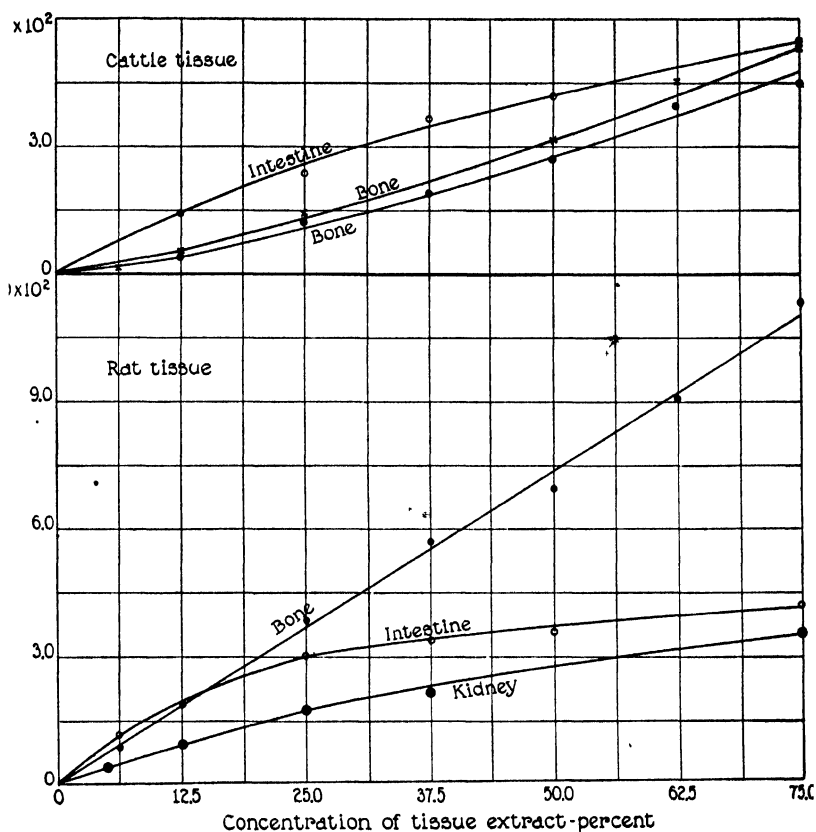


CHART 1. The relation between enzyme concentration ( $E$ ) and the observed phosphatase activity ( $Q$ ) as measured by the reciprocal of the time in minutes necessary to liberate 0.05 mg. of phosphorus per cc. of hydrolyzing mixture.

Northrop (15) suggested that the relative retardation in the activity of pepsin at high enzyme concentrations was due to the formation of an enzymically inactive compound of pepsin and the

products of protein hydrolysis. He was able to show that this combination proceeded according to the law of mass action.

The possibility that inorganic phosphate might, at higher enzyme concentrations, combine to form an enzymically inactive "phosphate-phosphatase" compound seems unlikely from an inspection of the inorganic phosphate contents of the various preparations studied. Thus the inorganic phosphate contents of the intestinal preparations, Preparations RIA and CIA, were 0.151 mg. and 0.061 mg. of phosphorus per cc. of enzyme solution, respectively, while those of the bone extracts, Preparations RBA and CBB, were 0.060 mg. and 0.017 mg. of phosphorus, respectively. Preparations RBA and CIA had practically the same concentration of inorganic phosphate, yet the former showed a direct proportionality of reaction velocity to enzyme concentration (with  $Q$  as a measure) whereas the latter did not.

Raising the inorganic phosphorus content of the rat bone extract to that of the intestinal preparation did not significantly decrease velocities at the higher enzyme concentrations. Thus the ratio ( $Q/E$ ) of the reaction rate ( $Q$ ) to the enzyme concentration ( $E$ ) at 12.5 per cent concentration of a rat bone extract (Preparation RBA) was  $0.144 \times 10^{-2}$  in comparison with  $0.138 \times 10^{-2}$  at 50 per cent and  $0.138 \times 10^{-2}$  at 75 per cent. When the concentration of inorganic phosphorus was raised to 0.166 mg. per cc. of extract, slightly in excess of that of a rat intestinal extract,  $Q/E$  fell from  $0.133 \times 10^{-2}$  at 12.5 per cent extract concentration to  $0.103 \times 10^{-2}$  at 75 per cent.  $Q/E$  for the intestinal extract, Preparation RIA, however, was  $0.144 \times 10^{-2}$  at 12.5 per cent and  $0.056 \times 10^{-2}$  at 75 per cent.

*Retardant Influence of Non-Protein Nitrogenous Substances*—The percentage of total nitrogen as non-protein nitrogen was much higher in the intestinal and kidney extracts than in the bone. This suggested that the lack of proportionality between reaction velocity and enzyme concentration in the intestinal and kidney preparations might be due to the products of tissue autolysis. Bradley (16) and Wells (17) have pointed out that the kidney and mucosa of the small intestine undergo rapid autolysis while skeletal, muscle, and connective tissues show only slight autolytic power.

1 volume of a rat intestinal extract (Preparation RIA), in-

activated by heating at 70–75° for 1 hour, and added to 2 volumes of bone extract (Preparation RBA) reduced the activity markedly. Before the addition of the inactivated intestinal extract,  $Q/E$  for extract concentrations of 12.5, 50, and 75 per cent were fairly constant. After the addition,  $Q/E$  fell from  $0.149 \times 10^{-2}$  at 12.5 per cent concentration of the bone extract to  $0.087 \times 10^{-2}$  at 50 per cent.

Peptone (Baker) in the concentration of 2 mg. per cc. of a rat bone extract, Preparation RBC, produced a similar retardant effect. Without peptone,  $Q/E$  at 12.5 and at 75 per cent were

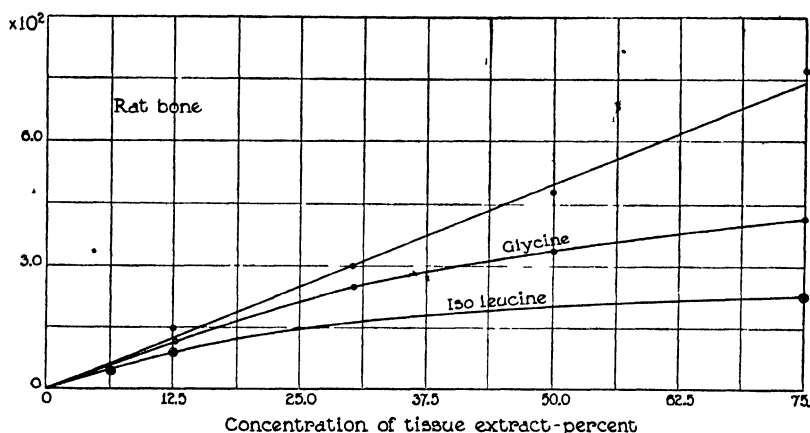


CHART 2. Effect of amino acids on the phosphatase activity of various concentrations of a rat bone extract.

identical,  $0.076 \times 10^{-2}$ . After the addition of the peptone,  $Q/E$  fell from  $0.081 \times 10^{-2}$  at 12.5 per cent to  $0.067 \times 10^{-2}$  at 75 per cent. Greater concentrations of peptone could not be used since precipitation resulted when the molybdate was added in the subsequent phosphorus determination.

The influence of amino acids (glycine and isoleucine) on the reaction velocity of a rat bone extract is shown in Chart 2. The concentration of glycine in the bone extract was 0.8 per cent; that of the *D*-isoleucine 1.39 per cent. The nitrogen concentration was of the same order as that found in rat intestinal extracts. Before the addition of the amino acids, the rat bone extract (Preparation

RBB), like the one (Preparation RBA) illustrated in Chart 1, showed excellent proportionality between extract concentrations, varying from 6.25 to 75.0 per cent, and reaction velocity. Both glycine and *D*-isoleucine, the only amino acids studied, markedly retarded the enzyme activity in the high enzyme concentrations while influencing activity at low concentrations, slightly, if at all.

The effect of removing the non-protein nitrogenous substances from intestinal extracts or the proportionality between enzyme concentration and reaction velocity was studied as follows:

The toluene was removed from a crude extract of cattle intestine. 0.1 *N* hydrochloric acid (about 25 cc. per 100 cc. of extract) was added slowly with stirring until maximum precipitation occurred. The mixture was centrifuged and the clear supernatant fluid discarded. The precipitate was washed in 95 per cent alcohol and then in ether, and dried in a vacuum desiccator. The yellowish brown powder was dissolved in 25 cc. of water with a small amount of sodium hydroxide.

100 cc. of crude cattle intestinal extract (Preparation CIA) yielded 13.5 mg. of dried powder.

Preparation	Dry weight per cc extract	Reaction velocity $Q \times 10^3$ at 12.5 per cent enzyme concentration	Reaction velocity Dry weight
	mg		
Crude	3 18	1 35	0 425
Purified	0 54	2 38	4 42

Calculated on a basis of dry weight, the purified preparation was 10.4 times as active as the unpurified.

The purified extract, kept at ice box temperature, retained its potency for 2 days. It deteriorated about 20 per cent in a week and about 30 per cent in 3 weeks. It contained very little non-protein nitrogenous material and very little inorganic phosphate.

This method of purification was successful in the case of the rat intestine only in fresh extracts. In preparations several weeks old practically no protein was precipitated with hydrochloric acid. Analyses showed that there was very little protein. Apparently the precipitation of protein in intestinal extracts is necessary for the precipitation of the enzyme.

Table II shows that removal of the non-protein nitrogenous substances from a crude cattle intestinal extract establishes proportionality between enzyme concentration and reaction velocity.

*Accelerating Effect of Magnesium*—The accelerating effect of magnesium on phosphatase activity, discovered by Erdtman (11) in 1927, has been studied by Hommerburg (10) and recently by Jenner and Kay (18). The latter found that, in general, the optimal concentration of magnesium for tissue phosphatases lay between 0.02 and 0.005 M.

TABLE II

*Comparison of Reaction Velocities of Crude and Purified Cattle Intestinal Extracts at Various Extract Concentrations*

$E$ = extract concentration	Crude extract (Preparation CIA <sub>1</sub> )		Purified extract (Preparation CIA <sub>1</sub> )		Crude extract (Preparation CIA <sub>2</sub> )		Purified extract (Preparation CIA <sub>2</sub> )	
<i>per cent</i>	$Q^* \times 10^3$	$(Q^*/E) \times 10^3$	$Q^* \times 10^3$	$(Q^*/E) \times 10^3$	$Q \times 10^3$	$(Q/E) \times 10^3$	$Q \times 10^3$	$(Q/E) \times 10^3$
6.25	1.20	0.192	0.51	0.082				
12.5					1.32	0.106	2.15	0.172
75.0	9.17	0.122	6.58	0.087	5.38	0.072	12.5	0.167

$Q^*$  is the reciprocal of the time (in minutes) necessary to liberate 0.03 mg. of phosphorus per cc. of hydrolyzing solution.

Preparation CIA<sub>1</sub> is a re-solution of the precipitate from 40 cc. of crude extract in 40 cc. of water. Preparation CIA<sub>2</sub> is a re-solution of the precipitate from 100 cc. in 35 cc. of water.

The rat extracts used in this study contained about 1 mg. per cent of magnesium; the cattle extracts from less than 0.1 to 0.4 mg. per cent (Table III).

The influence of magnesium was evaluated by studying its effect (1) upon the phosphatase activity in different extracts and at different enzyme concentrations and (2) upon the proportionality between enzyme concentrations and reaction velocity.

For these studies magnesium chloride was added to batches of rat bone extract (Preparation RBE) and cattle bone extract (Preparation CBD) so that they contained the following amounts of magnesium in mg. per 100 cc. of extract: 7.5, 33.4, 326, 1620. The reaction velocities were determined at the different enzyme concentrations of these extracts.

The length of time during which magnesium chloride remained in contact with the tissue extract (up to 48 hours) had no influence on the magnesium effect. The recorded magnesium concentrations represent the sum of the magnesium present in the preparations and the added magnesium. As was done by Jenner and Kay (18), the magnesium concentration is expressed as the negative logarithm of the molar concentration,  $q\text{Mg}$ .

TABLE III

*Comparison of Reaction Velocities of Various Tissue Extracts at Original and at Optimal Magnesium Concentration*

Source of extract	Mg* concentration in crude extract	Con- centration of extract in hydro- lysing mixture	Reaction veloc- ity of tissue extracts		$\frac{Q_2}{Q_1} \times 10^2$
			At original Mg con- centration	At optimal Mg con- centration	
	mg per cent	per cent	$Q_1 \times 10^2$	$Q_2 \times 10^2$	
Rat					
Intestine (Preparation RIE)		20.8	5.70	7.14	125
" ( " RID)	1.00				
Bone (Preparation RBE)	0.95	12.5	0.35	0.46	131
" ( " RBD)	0.82				
Kidney (Preparation RKE)	0.86	20.8	2.75	3.27	119
Cattle					
Intestine (Preparation CIA)	0.46	20.8	1.74	3.46	199
Bone (Preparation CBD)	0.40	25.0	1.43	2.08	145
" ( " CBB)	<0.10				

\* Magnesium was determined by the method of Briggs (19).

The results in Charts 3 and 4 and Table III illustrate the following: (1) the zone of optimal activity is a broad one at  $q\text{Mg}$  of 2.0 to 3.0 for all preparations studied (see also Jenner and Kay (18)) excepting cattle intestine which shows a sharp and narrow optimum at  $q\text{Mg}$  of 0.6 to 1.2; (2) the acceleration due to magnesium at optimal concentration varies from 25 to 100 per cent for the different enzyme preparations; (3) as the concentration of the rat or cattle bone extract is increased the magnesium optimum becomes narrower and shifts to the right (Chart 4);

(4) when the results illustrated in Chart 4 are plotted to show the relationship between the reaction velocity and the enzyme concentration,  $Q/E$  is constant in the extracts containing 7.5 mg. and 33.4 mg. of magnesium per 100 cc. of rat bone extract (Preparation RBE). In the extracts containing larger amounts of magnesium the reaction velocity decreases rapidly at the higher enzyme concentrations. In cattle bone extracts to which no magnesium has been added,  $Q/E$  increases as the concentration of the extract

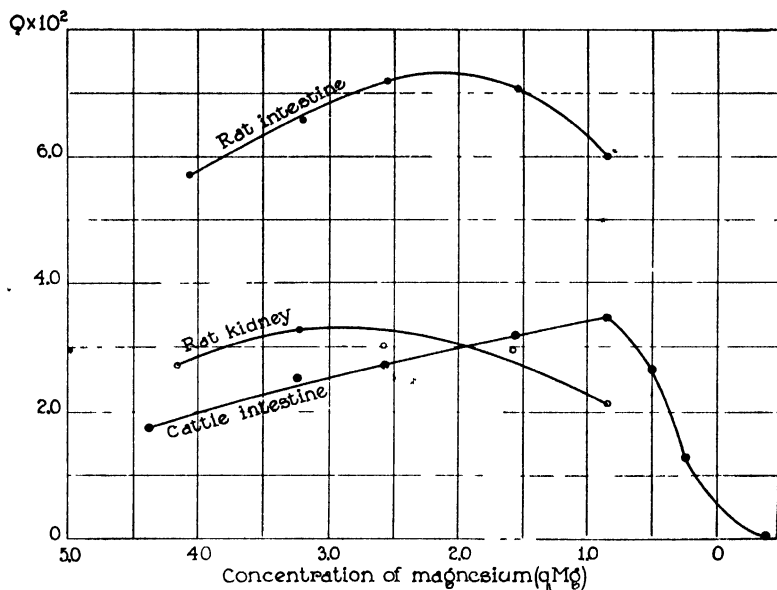


CHART 3. Optimal magnesium concentration curves for the phosphatase activity of various tissue extracts. Enzyme concentrations 20.8 per cent of hydrolyzing mixtures.

is raised (Chart 2). At a concentration of 6.9 mg. of magnesium per 100 cc. of extract, the values of  $Q/E$  at the lower concentrations approach more closely those at the higher concentrations of phosphatase, and in an extract of 32.2 mg. of magnesium per 100 cc., still more closely. In extracts containing higher concentrations of magnesium, the decreases in  $Q/E$  are of a smaller magnitude than those obtained with rat bone extract.

*Other Accelerants of Phosphatase Activity*—The finding that in



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cattle bone extracts the activities at the higher enzyme concentrations were relatively greater than at low enzyme concentrations suggested the presence of accelerating substances in the extracts.

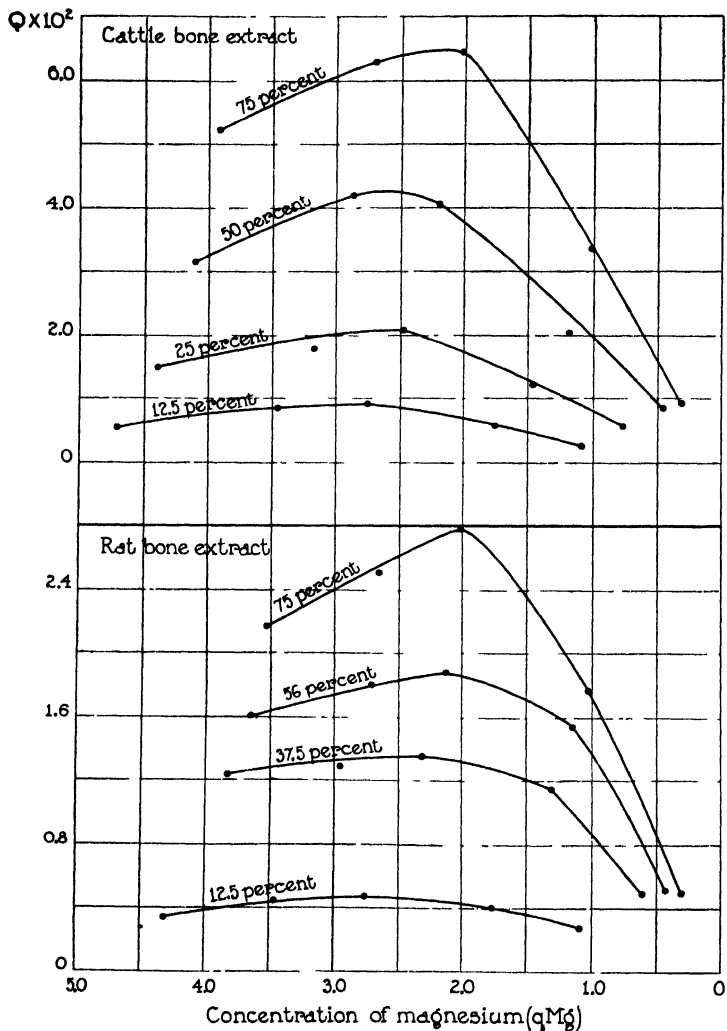


CHART 4. The effect of magnesium concentration on the phosphatase activity of varying concentrations of a rat bone and a cattle bone extract.

Control observations showed that the relatively low activity with dilute enzyme concentrations was not due to inactivation or to change in pH.

The presence of accelerating substances was investigated by determining the influence of cattle bone extracts, inactivated by heating at 75 to 80° for 1 hour, on phosphatase activity. 5.5 volumes of heated extract (Preparation CBD), added to 0.5 volume of enzyme increased the velocity ( $Q$ ) from 0.0009 to 0.0041, whereas an amount of magnesium equal to that in the added heated extract increased  $Q$  only to 0.0031. In a similar experiment with another heated extract (Preparation CBE) the activity was increased from 0.0018 to 0.0046, whereas an equivalent amount of magnesium increased  $Q$  only to 0.0024.

An aqueous extract of cartilage (cattle) was without effect. Heated cattle bone extract was dialyzed for 24 to 36 hours against water. The residue raised the velocity of a 6.25 per cent cattle bone extract at optimal magnesium concentration from an average value of 0.0036 to one of 0.0042. A trichloroacetic acid precipitate of cattle bone extract was also accelerating ( $Q = 0.0045$ ). An ether-alcohol extract of the precipitate, evaporated and redispersed in dilute sodium hydroxide, was without effect; the residue, redissolved in sodium hydroxide, raised the phosphatase activity to  $Q = 0.0051$ .

Apparently the substance or substances in heated cattle bone which accelerate phosphatase activity are either protein in nature or adsorbed to protein.

#### DISCUSSION

At present it cannot be said to what extent the enzymic activity of a tissue extract measures the enzymic activity of that tissue *in vivo*. Destruction of the tissue probably alters immediately the effects of local retardants or accelerants, substrate concentration, hydrogen ion concentration, and mechanisms for the diffusion of the reactant products. However, in so far as the activity of an extract is taken as representative of the activity *in vivo*, it is to be measured under certain standard and optimal conditions. In the present paper an attempt has been made to determine some of these conditions for tissue phosphatases so as

to permit the more accurate comparison of the activities of different tissues in the same or different organisms.

It was found that the course of the hydrolysis of glycerophosphate was not such as to allow the amount of phosphate liberated within a short initial period to be taken as a measure of the phosphatase concentration. Accordingly, the reciprocal of the time necessary to liberate a definite amount of phosphorus as phosphate was employed.

Intestinal and kidney extracts contain products of protein degeneration, the result of proteolytic autolysis, which retard markedly the phosphatase activity at the higher concentrations of enzyme. It follows that the activity of intestinal and kidney extracts may be compared only at those concentrations of phosphatase at which such retardation is minimal or lacking. At higher concentrations comparison may be made if the products of protein degradation are removed or if their effect is corrected for.

The magnesium content of various preparations varies widely. In view of the findings of Jenner and Kay (18) and also of those reported in this paper, comparison of the phosphatase activity of extracts without regard for this factor, is like the measurement of enzyme activity without regard for the pH; one is measuring the pH or the magnesium content quite as much as the concentration of phosphatase. The activities of different extracts should therefore be compared at optimal magnesium concentration. Moreover, it cannot be assumed that all phosphatase extracts will have the same magnesium optimum. In this study cattle intestinal phosphatase was found to have a distinctly different optimum from other tissue extracts studied. The presence of an optimal amount of magnesium does not alter the proportionality between reaction velocity and enzyme concentration.

In addition to the magnesium ion, the presence in sufficient concentration of other accelerant substances may be necessary to permit a valid estimation of activity. In cattle bone extracts, the activities at the lower concentrations are much less than called for by a direct proportionality between reaction velocity and enzyme concentration. The presence of an increased concentration of magnesium tended to restore the proportionality but in addition it was found that inactivated cattle bone extracts contain a thermostable, non-dialyzable, trichloroacetic acid-precipi-

table, and ether-alcohol-insoluble substance which accelerates activity at the lower phosphatase concentrations. The measurement of the activity of cattle bone extracts, or other extracts showing similar properties, at low concentrations should be corrected for the lack of this factor and of magnesium.

#### SUMMARY

1. When the reciprocal of the time necessary to liberate a certain amount of phosphate from glycerophosphate is used as the measure of phosphatase activity, the reaction velocity is directly proportional to the enzyme concentration only in rat bone extracts. In intestinal and kidney extracts of both rat and cattle the reaction velocity at higher concentrations of enzyme is less than called for by direct proportionality; in the cattle bone extracts, on the other hand, it is greater.

2. For the intestinal extracts, this lack of direct proportionality is due to the presence of products of proteolytic autolysis.

3. In the cattle bone extracts, the lack of direct proportionality is due to the absence, in sufficient concentration, of magnesium and one or more other accelerants.

4. The effect of the magnesium concentration upon (1) the relative activity of different extracts, (2) the activity of the same extract at varying enzyme concentrations, and (3) the proportionality between reaction velocity and enzyme concentration was studied.

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## STUDIES ON DIGESTIBILITY OF PROTEINS IN VITRO

### V. RATE OF LIBERATION OF CYSTINE ON HYDROLYSIS OF CASEIN. SOME OBSERVATIONS ON COLORIMETRIC TESTS FOR CYSTINE WHEN APPLIED TO PEPTIC AND ACID DIGESTS OF CASEIN\*

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This paper is a continuation of a series of studies carried on in this laboratory some time ago on the digestibility of proteins *in vitro* (1-3). The data now presented are the results of some preliminary experiments carried out in connection with proposed studies on the rate of liberation of amino acids from different proteins on digestion. It is known that on enzymic hydrolysis different amino acids are liberated at different rates. According to Abderhalden (4) gastric digestion does not liberate from proteins and peptones free amino acids. So called  $\alpha$ -biuret products are formed which contain high percentages of proline and phenylalanine. On the other hand, certain amino acids are readily liberated from proteins on pancreatic digestion, others are liberated more slowly, and still others are liberated from their combination with great difficulty. Tyrosine, tryptophane, and cystine are liberated very early in tryptic digestion. Proline and phenylalanine were found to be difficultly liberated, if at all, and glutamic acid was liberated fairly readily, but not so rapidly as tyrosine.

Fürth and Lieben (5), on the other hand, claim that tryptophane is not set free during the first stages of tryptic digestion. Ragins (6) found that after 1 hour's digestion with pancreatin, casein

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yielded three-fourths of its tryptophane, edestin little less than one-half, and squash seed globulin two-thirds of this amino acid. That proline is difficultly liberated from casein on tryptic digestion was shown by Hunter (7), who isolated an indigestible fraction having a high proline content. Dauphinee and Hunter (8) found that arginine was rapidly liberated from gelatin and from casein on tryptic digestion, but with edestin the rate was much slower. Histidine was found by Boone (9) to be readily split off from casein, edestin, and Witte's peptone when they were boiled for 1 hour with 5 per cent sodium hydroxide in absolute alcohol. He concludes accordingly that histidine probably occupies a terminal position in the molecule.

Notwithstanding the vast amount of literature on enzymic digestion of proteins, comparatively little is known on the rate of liberation of individual amino acids, and not all the statements are in agreement. Differences in the rate of liberation of different amino acids are very likely connected with peculiarities of molecular structure. Some amino acids may be more readily liberated than others because of differences in the way they are linked in the protein molecule, or because some may occupy a more exposed position in the molecule than others. The fact that certain amino acids in a given protein are liberated more readily than others does not justify the conclusion that these same amino acids can be equally readily liberated from all proteins. Conflicting statements in the literature as to the relative rate of liberation of different amino acids on digestion can well be explained on the ground that the observations were made on different proteins.

The methods which have been generally used in following the progress of the digestion of proteins have been limited largely to observations of physical changes in the substrate. The following are some methods which have been used: dyeing the protein and comparing the solution colorimetrically at different intervals as the protein dissolves; estimating the nitrogen which escapes precipitation with protein precipitants; electrical conductivity methods; determining nitrogen in the undigested portion obtained by precipitation with acids; noting liquefying changes in coagulated proteins; polarimetric determinations; viscosity determinations; formol titration; Van Slyke's amino nitrogen determination; and recently use has been made of the ultracentrifuge. Many of

these methods, although suitable for following advanced stages in the degradation of proteins, are not satisfactory for studying initial changes that occur. In general, they reveal only the extent of proteolysis rather than the nature of the structural changes that are taking place.

Data on the liberation of individual amino acids from proteins on hydrolysis have been obtained chiefly by isolation methods. The difficulties of quantitatively isolating amino acids when present in small quantities practically preclude the use of isolation methods for measuring their rates of liberation.

Recently developed colorimetric methods for the determination of certain amino acids appeared to offer a means whereby free amino acids or groups reactive to the colorimetric reagents might be determined in the initial stages of digestion or hydrolysis. Data on this subject would be of value not only nutritionally from the standpoint of getting information on the digestibility of different proteins, but also in connection with problems related to molecular structure.

Because of the great interest which has recently developed in cystine, and because of the colorimetric methods available for its determination, this amino acid was chosen as the first to be investigated in a series of studies on the rate of liberation of amino acids.

The work described in this paper was carried on with casein. The numerous investigations which have been made on the acid and the enzymic hydrolysis of casein show that it is more readily acted upon by hydrolytic agents than most other proteins which have been studied. The remarkable susceptibility of casein to the effect of different reagents, particularly alkali, which is commonly used in its preparation, probably accounts for some of the conflicting data in the literature on its composition.

*Material*—The casein used was prepared from fresh skim milk according to the method of Van Slyke and Baker (10). It was precipitated at 15°, and at no stage of its preparation, including the final drying, did the temperature exceed 18 or 20°. The low concentration of acid used, the entire avoidance of alkali, and the low temperature at which the material was held make it improbable that the protein suffered any material change during its preparation. It had the following percentage composition: ash, 0.02;



moisture, 7.76; nitrogen, 15.84; cystine, 0.33, as determined both by Folin and Marenzi's method and by that of Sullivan.

The cystine was prepared in the laboratory, and was of established purity. Fairchild Brothers and Foster's pepsin was used in the digestion experiments.

*Acid Hydrolysis of Casein*—Samples of casein were hydrolyzed for periods ranging from 15 minutes to 24 hours, and the hydrolysates were examined both by the Folin and Marenzi (11) and by the Sullivan (12) methods for the colorimetric determination of cystine.

For the determinations by the Folin and Marenzi method samples of casein equivalent to 1 gm. of the ash- and moisture-free material were heated in 20 cc. of 20 per cent hydrochloric acid to gentle boiling for the desired length of time. The hydrolysates were diluted at once with distilled water to a volume of 50 cc. and decolorized with kaolin. 5 cc. aliquots were taken and the color developed by the Folin and Marenzi reagents compared against that similarly developed with a standard cystine solution. The color values obtained are shown on Chart I as percentages of cystine calculated on the basis of the ash- and moisture-free casein.

Two striking features of the results are (1) the exceedingly high values obtained on the short periods of hydrolysis, beginning with 0.46 per cent after 15 minutes and reaching a maximum of 0.55 per cent after 45 minutes, and (2) the abrupt drop to 0.42 per cent after a 1 hour's hydrolysis. Thereafter the values gradually decreased, reaching 0.33 per cent at the end of 18 hours, which then remained constant up to 24 hours. This constant value is practically identical with the cystine content of the casein as determined by the Sullivan method. The high values found by the Folin and Marenzi method on the shorter periods of hydrolysis must be attributed to some causative factor other than free cystine.

For the determinations of cystine by the Sullivan method samples of casein equivalent to 1 gm. of ash- and moisture-free material were used when the periods of hydrolysis were 6 hours or longer. Because of the low cystine content of casein it was found necessary to take 2 gm. for hydrolyses of shorter duration than 6 hours in order to get a color intensity satisfactory for accurate measurements. The results of these determinations are given in Chart I.

The results obtained on the shorter periods of hydrolysis are in striking contrast to those obtained by the Folin and Marenzi method. Instead of the high values reaching a maximum at the end of 45 minutes and then dropping abruptly, the Sullivan method shows that gradually increasing quantities of cystine are liberated until a maximum of 0.33 per cent is reached after 6 hours hydrolysis. On further hydrolysis this value remains constant up to 24 hours, and is the same as the value obtained by the Folin and Marenzi method after 18 hours hydrolysis.

The values obtained by the Sullivan method show that cystine is early liberated from casein on acid hydrolysis. After a 30 minute hydrolysis, 20 per cent of the total cystine content of the protein had been liberated, and after  $3\frac{1}{2}$  hours the value amounted to 50 per cent.

*Digestion of Casein with Pepsin*—Although it is generally accepted that free amino acids are not liberated when proteins are digested with pepsin, it was thought desirable as preliminary to a study of the rate of liberation of cystine when casein is acted on by digestive ferments, to apply the colorimetric cystine tests to peptic digests. Because of the inadequacy of the methods heretofore used for estimating cystine when present in small quantities it is not inconceivable that in previous studies some of this amino acid which may have been present escaped detection.

The digestions were made separately on duplicate samples of casein, each equivalent to 2 gm. of the ash- and moisture-free protein. The casein was added to a mixture of 25 cc. of 0.1 N hydrochloric acid and 25 cc. of a 0.2 per cent solution of pepsin in 0.1 N hydrochloric acid. Before they were mixed, all the components of the digest were separately heated to 38°. The mixtures were then placed at once in an incubator and maintained at 37–38° for periods ranging from 5 minutes to 18 hours. At the end of the digestion periods the digests were heated to 80° in order to inactivate the pepsin, and then diluted with distilled water to 100 cc. Duplicate 5 cc. portions were taken and examined according to both Folin and Marenzi's and Sullivan's methods for the estimation of cystine. The color developed was compared with that similarly produced in a standard cystine solution.

The tests made by the Sullivan method on the peptic digests after different periods of digestion gave negative results in every

case, even after 18 hours digestion. These results are in agreement with the views generally held that free amino acids are not liberated on peptic digestion.

In every determination made by the Folin and Marenzi method a blank test was run on the pepsin by incubating in separate flasks simultaneously and parallel with the casein-pepsin mixtures, 25 cc. of a 0.2 per cent solution of pepsin in 0.1 N hydrochloric acid, and determining the color developed with the Folin and Marenzi reagents. The color values thus obtained were subtracted as a blank from those obtained for the casein digests. The corrected cystine equivalent values found for the casein digests are given in Chart I.

The values obtained by the Folin and Marenzi method for both the peptic digests and the acid hydrolysates follow the same general trend. They are in striking contrast to those obtained by the Sullivan method. There is first a rapid rise in values, followed by a sudden drop, then a decrease until a constant value is reached. The peptic digests starting with a cystine equivalent value of 0.21 per cent after 5 minutes digestion reached a maximum of 0.40 per cent after digestion for 1 hour, then dropped to 0.25 per cent in 5 hours. On further digestion this value remained constant. With the more specific Sullivan method, on the other hand, the peptic digests gave negative results, whereas the acid hydrolysates showed continually increasing values until a maximum was reached which thereafter remained constant.

The color values obtained by the Folin and Marenzi method after short periods of digestion raised the question whether we were measuring the effect of enzyme action or that of mild acid hydrolysis. The experiments outlined in Table I were designed to get information on this question, and also to throw light on the nature and manner of formation of the chromogenic factor or factors responsible for the color values obtained. The expression "casein-pepsin mixture" as used in Table I refers to a mixture of 2 gm. of casein in 25 cc. of 0.1 N hydrochloric acid and 0.2 per cent solution of pepsin in 0.1 N hydrochloric acid. The "casein-hydrochloric acid mixture" consisted of 2 gm. of casein in 50 cc. of the acid. In the first four experiments essentially the same values were obtained, irrespective of whether pepsin was used or not. In Experiment 7, the same value, 0.24 per cent, was ob-

tained by merely suspending casein in distilled water for 2 hours at room temperature as was obtained by incubating casein with 0.1 N hydrochloric acid for 1 hour (Experiment 6). Other experiments showed that incubation of the casein with hydrochloric acid for longer periods, up to 3 hours, did not increase the chromogenic value of the casein digest. It appears, therefore, that up to values of 0.24 per cent neither the hydrochloric acid nor the pepsin used had any significant part in the production of the factor or factors responsible for the characteristic color developed by the Folin and Marenzi reagents.

TABLE I

*Color Values Obtained by Folin and Marenzi's Method on Casein under Different Treatments*

Experiment No.	Description	Color values
		<i>per cent</i>
1	Casein-pepsin mixture heated to 80°, tested at once	0 20
2	Casein-HCl mixture heated to 80°, cooled, tested at once	0 20
3	Casein-pepsin mixture tested at once without heating to 80°	0 19
4	Casein-HCl mixture tested at once without heating	0 18
5	Casein-pepsin mixture allowed to stand 24 hrs. at room temperature	0 21
6	Casein-HCl mixture incubated 1 hr. at 35-38°	0 24
7	Casein suspended in distilled water 2 hrs. at room temperature. Tests made directly on suspension	0 24

The color values obtained with the peptic digests evidently do not represent free cystine, but are produced by some compound or compounds other than cystine, or by certain reactive groups in the casein which are exposed during the initial stages of proteolysis.

Determinations were made of disulfide groups, —S—S—, both on peptic digests and acid hydrolysates in order to see whether the color values found by the Folin and Marenzi method could be ascribed to exposed disulfide groups in the partial cleavage products of the casein and, if so, to note any correlations between the disulfide values and those obtained by the tests for cystine made by the colorimetric methods already described. The determinations were made by the sodium nitroprusside method as described

by Walker (13). The color developed was compared with an empirical standard consisting of a mixture of Bordeaux red and methyl orange in the proportions given by Abderhalden and Wertheimer (14). This mixture had been previously standardized colorimetrically against a cystine solution of known concentration containing a few drops of sodium nitroprusside to which 5 drops of a 10 per cent sodium cyanide solution had been added. The empirical dye standard was used instead of a standard cystine solution because the latter gives with sodium nitroprusside a color which is so unstable as to make an accurate reading rather difficult.

TABLE II

*Relative Values of Disulfide Groups in Acid Hydrolysis of Casein, Expressed As Percentages of Cystine*

Length of hydrolysis	Disulfide values	Length of hydrolysis	Disulfide values
	<i>per cent</i>	<i>hrs</i>	<i>per cent</i>
3 min.	0 29	5	0 56
15 "	0 31	6	0 58
30 "	0 45	8	0 58
45 "	0 48	10	0 57
1 hr.	0 50	12	0 55
2 hrs.	0 51	18	0 50
3 "	0 51	24	0 50
4 "	0 54	36	0 49

Tests for reactive disulfide groups in casein digests after digestion with pepsin for periods ranging from 5 minutes to 18 hours gave faint but decisive results.

For the nitroprusside tests on the acid hydrolysates, samples of casein equivalent to 1 gm. of the ash- and moisture-free protein were heated in 20 cc. of gently boiling 20 per cent hydrochloric acid for various periods. The values are given in Table II. For the sake of convenience only, the results are expressed in percentages of cystine. They are not intended to be considered as actually representing cystine, for several compounds other than cystine give the nitroprusside reaction (15). Although the values obtained are much higher, they run roughly parallel with the cystine values found for the acid hydrolysates by the Sullivan method. Increasing values were found, which reached a maxi-

mum after a 6 hour hydrolysis. On the other hand, there is no correlation between the nitroprusside values and those obtained by the Folin and Marenzi method. The maximum values occurred in the Folin and Marenzi determinations within the 1st hour of hydrolysis, followed by a sudden drop. No corresponding drop was indicated in the disulfide values.

The trend of the values obtained by the Folin and Marenzi method, as shown in Chart I, indicates that more than one chromogenic factor is involved: (1) The characteristic sudden rise and fall in the values obtained suggest the rapid formation of some

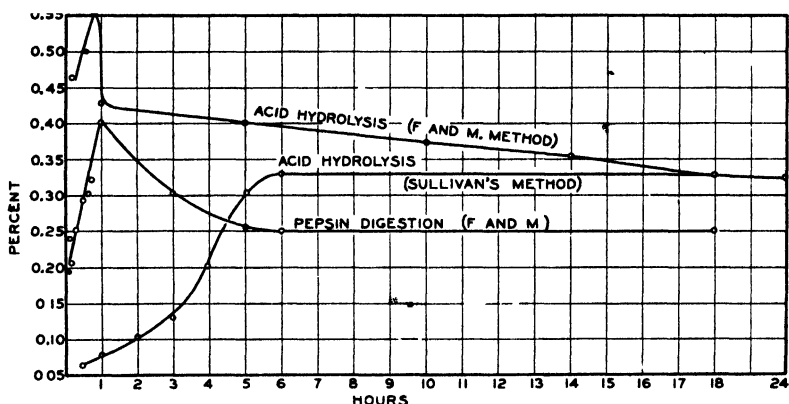


CHART I. Color values of hydrolysates and peptic digests of casein as determined by Folin and Marenzi's and Sullivan's methods for estimating cystine.

reactive compound or compounds which is either soon volatilized or converted into products less reactive toward the colorimetric reagents; (2) on the other hand, the values obtained in the experiments outlined in Table I and the constant value obtained for the peptic digest after 5 hours digestion indicate the presence of a relatively stable type of a chromogenic compound or compounds, contributing a cystine equivalent value of about 20 to 25 per cent. It has been pointed out (16) that there are substances other than cystine which may well be present in protein hydrolysates, such as levulinic acid, pyruvic acid, furfural and some of its derivatives, which also react with the Folin and Marenzi reagent. A com-

pound such as one of these may account for the more stable chromogenic factor referred to.

The rapid rise in values reaching a maximum after 1 hour's digestion with pepsin and after about  $\frac{3}{4}$  hour's hydrolysis with hydrochloric acid, and the subsequent drop to a constant value, are not due to the formation of volatile substances, but apparently to exposed reactive groups in the partial cleavage products, which after reaching a maximum, are rapidly converted into non-reactive compounds. Evidence supporting this view was obtained in another study, the details of which will be published soon. Casein was digested with pepsin for 1 hour. Three fractions were isolated from the digest having strikingly different properties and composition. Each of these fractions was tested separately by the Folin and Marenzi method. The sum of their chromogenic values was found to have a cystine equivalent value of 0.40 per cent, which is the same as the maximum found in the peptic digest at the end of 1 hour's digestion, as shown in Chart I.

#### SUMMARY

Colorimetric estimation of cystine by the Sullivan method showed that cystine is early liberated from casein on acid hydrolysis (20 per cent hydrochloric acid). After a 30 minute hydrolysis, 20 per cent of the cystine content of the casein was liberated, and after  $3\frac{1}{2}$  hours the value amounted to 50 per cent. No increase in cystine liberated was obtained after 6 hours hydrolysis, the value remaining constant at 0.33 per cent. When tested by the Folin and Marenzi method for estimating cystine, the acid hydrolysates of casein showed at first abnormally high values with a striking rise and fall in chromogenic values. A rise from 0.46 per cent after a 15 minute hydrolysis to 0.55 per cent after 45 minutes was followed by an abrupt drop within 15 minutes to 0.42 per cent. The values then slowly decreased on further hydrolysis, becoming constant at 0.33 per cent at the end of 18 hours.

Tests made by the Sullivan method on peptic digests of casein showed that no detectable quantity of cystine was liberated. This is in accord with the generally accepted view that free amino acids are not liberated on peptic digestion. With the Folin and Marenzi method, however, an early rise and fall in values occurred similar to that observed with the acid hydrolysates. The values

increased from 0.21 per cent after digestion for 5 minutes to 0.40 per cent at the end of 1 hour. Thereafter the values gradually decreased, becoming constant at 0.25 per cent after 5 hours digestion.

The color values obtained with the peptic digests are produced by some compound or compounds other than cystine, or by certain reactive groups in the casein which are exposed during the initial stages of proteolysis. Evidence is presented showing that at least two factors are involved.

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## FURTHER STUDIES ON THE DETOXICATION OF PHENYLACETIC ACID

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In the previous work done on the detoxication of phenylacetic acid in this laboratory, we have never been able to account for more than half of it as the glutamine conjugate in the human urine. We ascribed this to either (a) conjugation with other substances besides glutamine, (b) an incomplete detoxication, or (c) the methods of analysis.

### *Conjugation with Glucuronic Acid*

Quick (1) believed that glucuronic acid may arise not from glucose itself, but from glycogen and sugar-forming amino acids when glucuronic acid is needed for detoxication. In his experiments depancreatized dogs were able to produce glucuronic acid the same as normal dogs, but under these conditions glucuronic acid is produced at the expense of glucose which would otherwise appear in the urine.

The ratio between the conjugation of benzoic acid with glucuronic acid and glycine in the case of animals was also determined by Quick (2). The results show that the amount combined with glucuronic acid in these cases was by far larger than had previously been suspected.

Brakefield (3) finds only traces of or no glucuronic acid after feeding benzoic acid to humans while Quick (4) under the same conditions reports 11 per cent conjugation. However, when phenylacetic acid is fed to dogs (2), it is detoxicated in a manner similar to benzoic acid.

*Incomplete Detoxication*

Human subjects were fed phenylacetic acid; the urine was acidified and extracted with various organic solvents, but no free phenylacetic acid was found.

*Experiment 1*—The object of our experiment was to determine to what extent, if any, glucuronic acid is employed by the human

TABLE I  
*Rate of Glucuronic Acid Excretion after Feeding Varying Amounts of Phenylacetic Acid*

Date	Amount fed	Phenylacetic acid eliminated		Date	Amount fed	Phenylacetic acid eliminated	
		Per cent of total	Per cent combined with glucuronic acid			Per cent of total	Per cent combined with glucuronic acid
	gm				gm		
Nov. 19, 23	1	93 0	0 0	Jan 27	7	99 1	4 5
" 30, Dec 1-4	2	97 5	0 0	" 30	8	99 1	6 8
Dec 5-8, Jan 4, 5	3	98 0	1 0	Feb 1	8	98 4	5 7
Jan 6, 7, 10	4	97 9	4 0	" 2	8	97 4	5 0
" 11	5	97 0	4 9	" 3	10	98 8	5 3
" 12	5	98 2	4 6	" 6	10	98 7	5 2
" 13	5	98 5	3 5	" 8	10	99 5	5 3
" 14	5	98 4	6 4	" 9	10	98 4	5 3
" 16	5	98 2	7 1	" 10	10	98 9	5 2
" 17	5	98 4	4 9	" 11	10	99 0	5 6
" 18	5	98 4	4 9	" 13	8	98 5	5 6
" 20	6	98 2	5 6	" 15	8	98 5	5 2
" 21	6	98 6	5 7	" 16	7	98 5	5 1
" 23	6	97 9	6 6	" 17	7	98 0	5 5
" 25	7	98 9	5 7	" 23	6	98 7	6 5
" 26	7	99 0	5 4	" 24	6	99 3	6 8

subject for detoxication of phenylacetic acid and the conditions under which this might take place.

In these experiments phenylacetic acid was fed to human subjects as the sodium salt in increasing daily doses in order to place a continual and ever increasing strain on the defense mechanism of the body, in the hope that in this way we might overtax the

power of the body to produce glutamine and force it to resort to a glucuronic acid detoxication. Table I shows the results obtained.

*Experiment 2*—Small doses of phenylacetic acid, 3 gm., were fed to thirty-four normal students and the urine was collected hourly over a period of 5 hours. It was thought that the small dose might be detoxicated by means of glucuronic acid and if so we wished to ascertain the rate at which this combination took place. It seemed quite possible to us that small amounts of phenylacetic acid excreted during the early stage of detoxication

TABLE II

*Glucuronic Acid Excretion after Feeding Phenylacetic Acid*

5 gm. were given every 8 hours, a total of 45 gm. in 72 hours.

Time of feeding	Phenylacetic acid eliminated	
	Total	Combined with glucuronic acid
	gm	gm
9 a.m.	1 84	0 0
5 p.m.	4 74	0 807
12 "	4 33	0 252
9 a.m.	2 68	0 602
5 p m	3 40	1 515
12 "	3 89	0 384
9 a.m.	3 34	0 301
5 p.m.	4 36	1 566
12 "	6 10	0 231
Total . . . . .	34 68	5 658

79.29 per cent of the total phenylacetic acid fed was recovered. 16.00 per cent of the total eliminated was conjugated with glucuronic acid. 12.40 per cent of the total fed was conjugated with glucuronic acid.

might have been overlooked, when large volumes of urine were collected over a period of 24 hours. The results of the experiment, however, may be considered entirely negative as five subjects of the thirty-four showed less than 1 per cent of glucuronic acid conjugation at one time or another, and the others showed less or none at all.

*Experiment 3*—This experiment was undertaken in much the same fashion, except that the subject was fed 5 gm. of phenylacetic acid every 8 hours for a period of 72 hours, a total feeding of

45 gm., and the amount of glucuronic acid and phenylacetic acid determined at the end of each 8 hour period. (See Table II.)

*Methods*—Phenylacetylglutamine was prepared by the usual method (5). Total phenylacetic acid was determined by the method of Kingsbury and Swanson (6) as used by them for the determination of total benzoic acid, except that, instead of using quantities of urine as described in their method, the quantity varied inversely with the amount of phenylacetic acid ingested. Glucuronic acid was determined by its reducing power towards Somogyi's reagent (7).

From this work it is clearly seen that glucuronic acid plays a rather small rôle in the detoxication of phenylacetic acid in the human as compared with similar work on animals except when excessive amounts are ingested. Thus Quick (2) showed that phenylacetic acid is excreted by the dog, conjugated with glucuronic acid to the extent of about 34 per cent, the other 66 per cent appearing as the glycine conjugate. Table I shows that after repeated ingestions of phenylacetic acid at 24 hour intervals the body is still capable of furnishing glutamine for detoxication purposes and that the amount of glucuronic acid produced rarely exceeds a 5 per cent conjugation.

However, the same or smaller doses, but ingested at 8 hour intervals, required considerable amounts of this substance, as Experiment 3 (Table II) shows, but we were unable to complete the series as the subject became nauseated on account of the repeated ingestions. During the second period we obtained greater glucuronic acid conjugation, indicating the body's inability to synthesize sufficient glutamine, thus resorting to glucuronic acid conjugation. Phenylacetic acid retention was also observed.

#### *Methods of Analysis of Phenylacetylglutamine*

Folin and Flanders (8) have noticed that hippuric acid in the urine is appreciably hydrolyzed while evaporating on a water bath, so it is to be expected that phenylacetylglutamine, which is quite unstable, would also be destroyed to a considerable extent. In a series of four experiments, assuming that all the phenylacetic acid ingested should appear as a glutamine conjugate, an average of 51 per cent of phenylacetylglutamine was recovered from the urine. To check this by a recovery experiment, 16.4 gm. of

phenylacetylglutamine were dissolved in 2 liters of normal urine and the urine was treated in the usual way (5). Only 47.4 per cent of the original amount was recovered. Therefore, it seems that the large amount of phenylacetylglutamine unaccounted for is not to be ascribed to any extensive conjugation with any other substances, but rather to the losses inherent in the evaporation, as the extraction itself seems to give quantitative removal of the phenylacetylglutamine once it is in the apparatus.

In connection with this work we were able to get an approximate estimate of the time required for the body to detoxicate phenylacetic acid. A total of 20 gm. (in four portions) was ingested by two subjects, each taking 5 gm. in two doses 12 hours apart; 12 hour volumes of the urine, each including both night and day specimens, were evaporated and extracted; and the first 12 hour sample was compared with the following 12 hour sample. It was found that in the urine excreted during the first 12 hours following ingestion of the acid, 53.8 per cent of the theoretical phenylacetylglutamine was recovered, and in the second 12 hour period only 1.4 per cent. Thus over 95 per cent of the phenylacetic acid is taken care of by the body during the first 12 hours and less than 5 per cent remains to be detoxicated in the second 12 hours. Hence, whether we approach this detoxication problem from the glutamine conjugate or from that of glucuronic acid, the ratio of one to the other is very nearly the same, that is about 18:1.

In aqueous solution, pure phenylacetylglutamine shows a fairly strong acidity. Its hydrogen ion concentration, measured by indicators and checked approximately electrometrically, for 0.1 N solution is  $6.9 \times 10^{-3}$ , corresponding to an ionization of about 7 per cent, and for 0.01 N solution,  $2.6 \times 10^{-3}$ , corresponding to an ionization of about 25 per cent. Its warm 0.1 N solution decomposes the carbonates of alkalies and alkaline earths, also those of lead and copper. Its salts thus far studied are all soluble in water, making separation by precipitation thus far impossible. The insolubility of the barium salt in absolute alcohol (0.87 gm. per liter) is used to separate it from urea; the presence of 5 per cent of water in the alcohol raises its solubility over 400 per cent.

*Decomposition by Evaporation*—Two samples were dissolved in 25 cc. of water in small Kjeldahl flasks which were immersed in boiling water and a slow current of air passed through (over 2½

hours), and run into hydrochloric acid 0.5024 N. The results were as follows:

Substance	Acid neutralized	Decomposition
gm.	cc.	per cent
0 1632	0 26	21 1
0 1625	0 32	26 1

This percentage decomposition is based on the assumption that the phenylacetylglutamine lost 1 molecule of ammonia and was converted into phenylacetylglutamic acid. A similar experiment run at 60° showed only one-fourth as much decomposition.

*Decomposition of Phenylacetylglutamine on Evaporation with Alkaline Solutions*—Experiments were conducted as above; in one case 50 cc. of saturated barium hydroxide solution were taken and boiled off slowly during 1 hour into standard acid kept in an ice bath; in the other case N KOH solution was used. The results were as follows:

Substance	Acid neutralized	Decomposition
gm.	cc.	per cent
0 2002	8 80 (0 0894 N)	104 0
0 2015	7 48 (0 1014 N)	99 5

Boiling with barium hydroxide solution for 15 minutes caused 71 per cent decomposition, and even in the cold there is a slight loss of ammonia on standing.

*Determination of Phenylacetylglutamine by Means of Its Amino Nitrogen*—Small samples of phenylacetylglutamine (0.1266 gm. in each case) were boiled 2 hours under a reflux with 10 cc. of 62 per cent sulfuric acid. The mixture was cooled, diluted, made alkaline with sodium hydroxide, and boiled until all the ammonia was liberated, then evaporated to dryness on the steam bath, and the residue dried in a vacuum oven overnight at 55–60°. The dry residue was ground up and extracted with 95 per cent alcohol made faintly acid with sulfuric acid. This alcoholic solution was concentrated to small volume, run into the Van Slyke apparatus, and the amino nitrogen determined, after 10 minutes shaking.

1 cc. of  $N_2$  = 0.0118 gm. of phenylacetylglutamine. The results were as follows:

N* cc.	Yield per cent
10 52	98 0
10 12	94 4
11 79	109 9
11 70	109 0

\* Normal temperature and pressure corrected for blank.

These figures give an average yield of 102.8 per cent. Using phenylacetylglutamine without previous hydrolysis, we observed on two samples 2.17 and 3.20 per cent decomposition, average 2.69 per cent, after 10 minutes shaking.

#### SUMMARY

1. The apparently incomplete conjugation of phenylacetic acid with glutamine when it is ingested by the human subject in moderate doses may best be ascribed to the hydrolysis of phenylacetylglutamine when the urine containing it is evaporated on the water bath.

2. About 95 per cent of the phenylacetic acid ingested in moderate doses by the human subject is detoxicated with glutamine, and about 5 per cent with glucuronic acid. On continued ingestion the ratio is shifted in favor of the latter.

3. Some properties of phenylacetylglutamine are enumerated.

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# THE EFFECT OF THE FEEDING OF FLUORIDES UPON THE CHEMICAL COMPOSITION OF THE TEETH AND BONES OF ALBINO RATS

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The toxic effect of fluorides upon the teeth and bones of animals has been demonstrated. In 1925 McCollum, Simmonds, Becker, and Bunting (1), also Schulz and Lamb (2), reported a disturbance in the quality and structure of the teeth of albino rats, produced by amounts of fluorides only slightly above that occurring in natural foods.

Since then the observed harmful effects of the feeding of rock phosphate as the source of calcium and phosphorus to various animals (3-7) has been related to its fluorine content by Tolle and Maynard, 1928 (5), Taylor, 1929 (6), and Bethke, Kick, Edgington, and Wilder, 1929 (7).

More recently the writers (8, 9) have shown that the presence of low concentrations of fluorides in drinking water is the cause of the peculiar human dental defect known as "mottled enamel" which occurs in many parts of the world. A defect similar if not identical in characteristics has been produced in rats, in guinea pigs, and in dogs (10) by the feeding or injection of fluorides.

As a result of the feeding of fluorides, the teeth not only lose the pigment and translucency normal to them, becoming dull and chalky white in appearance, but the enamel corrodes and chips off. Over longer periods of feeding there is a marked tendency to overgrowth or excessive wearing down of the incisors of rats. The severity of the defect is in proportion to the amount of fluorine fed. All of these changes indicate alterations in the tooth structure and composition.

Some work has been done to determine the nature of the changes.

An increase in the fluorine content of the teeth and bones of animals fed fluorides has been demonstrated in dogs by Sonntag (11), in pigs by Bethke and coworkers (7), and in guinea pigs by Christiani and Gautier (12). Bethke also analyzed the bones of the fluoride-fed pigs for their ash, calcium, and phosphorus content and reported a general tendency toward a decrease in the ash content of the fluoride-fed animals but no significant difference in the calcium and phosphorus content of the ash.

McClure and Mitchell (13), on the other hand, as a result of their study of the effect of fluorides upon the metabolism of calcium, and upon the chemical composition of the bones, report an average increase of 1.3 per cent in the ash content of the bones and an average decrease of 1.05 per cent in the calcium content of the ash of the bones with a resulting depression of the calcium to phosphorus ratio when fluorine as sodium fluoride was fed at 0.03 or 0.06 per cent of the ration. Calcium fluoride in the same concentration was not consistently observed to have the same effect, however. An apparent difference in susceptibility to dietary fluorine between the humerus and the femur was also reported.

The findings of these latter workers would suggest that the toxic effect of fluorides on bone development is due at least in part to its interference with the deposition of calcium in the bone. They explain the increase in ash content on the basis of the deposition of an abnormal non-calcium constituent of the bone, "probably a fluoride of another mineral." No analyses of teeth were made by these workers.

In 1932, this laboratory reported the preliminary results of an investigation of the effect of fluoride feeding upon the chemical composition of the incisor teeth of rats at the March meeting of the International Association for Dental Research (14). This paper reports more fully the effect of the feeding of fluorides in different concentrations upon the calcium, phosphorus, and ash content of the incisor teeth and also of the bones (tibias) of albino rats.

Hauck, Steenbock, and Parsons (15) have more recently reported (1933) that the addition of 0.15 per cent sodium fluoride to the diet of young rats produced a variable effect upon the ash content of the bones depending upon the calcium content of the

diet. However, the total ash content of the incisor teeth was decreased in all cases. They did not make calcium and phosphorus analyses.

#### EXPERIMENTAL

Albino rats taken at the time of weaning (28 days) were placed upon the adequate basal ration, Sherman's Diet B (16), in which 0.05 or 0.1 per cent of sodium fluoride was incorporated. Litter mates were continued as controls upon the same basal ration without the addition of sodium fluoride. Sodium fluoride fed at the 0.05 percentage level has previously been demonstrated (10) to produce outward changes characteristic of fluoride injury in the incisors of rats and yet is not of sufficiently high concentration to interfere with the general health of the animals except for slight stunting of their growth. When as much as 0.1 per cent of the ration is sodium fluoride, there is marked inhibition of growth of the rat.

The animals were kept in square galvanized iron cages and given the ration and distilled water *ad libitum*. At the end of experimental periods of 60 and 120 days the animals were chloroformed and the incisors and left tibia of each animal were dissected free from flesh which had been loosened by boiling in distilled water for 5 minutes.

One upper and one lower incisor and one tibia from each rat were used for chemical analyses. In order to reduce the percentage of error of the chemical determination and reduce the effect of individual variation which was evident from preliminary analyses of the teeth of individual rats, composites were made of the teeth or tibia of four rats and prepared for analyses as follows: The teeth were extracted for two 9 hour periods with fresh, boiling 95 per cent alcohol, followed by two 9 hour extraction periods with boiling anhydrous ethyl ether. The fat-free material was then dried in a Freas electric oven at 100° and weighed to constant weight to obtain the absolute dry weight of the tooth. The time required for reaching constant weight varied from 1 to 2 weeks. It was noted that the teeth of the fluoride-fed animals reached a constant dry weight in a shorter period of time than those of the control animals.

The teeth were then ashed in a muffle furnace at approximately

TABLE I  
Average Composition of Incisor Teeth of Rats Fed Basal Ration with and without Addition of Sodium Fluoride

Dietary supplement	Length of test period	No of rats represented	Weight of dry incisors (1 upper, 1 lower)	Ash in dry incisors $\pm$ p e *	Ca in dry incisors $\pm$ p e	Ca in ash $\pm$ p e	P in dry incisors $\pm$ p e	P in ash $\pm$ p e	Ca P ratio $\pm$ p e
				per cent	per cent	per cent	per cent	per cent	
None (control series)	60	48	0 1164	77 7 $\pm$ 0 06	29 2 $\pm$ 0 32	37 7 $\pm$ 0 36	15 1 $\pm$ 0 11	19 3 $\pm$ 0 16	1 95 $\pm$ 0 01
	120	44	0 1448	78 1 $\pm$ 0 19	28 6 $\pm$ 0 17	37 8 $\pm$ 0 23	14 7 $\pm$ 0 08	19 1 $\pm$ 0 05	1 93 $\pm$ 0 02
0 05 per cent sodium fluoride	60	44	0 1123	77 4 $\pm$ 0 10	29 0 $\pm$ 0 24	36 9 $\pm$ 0 26	14 9 $\pm$ 0 54	18 9 $\pm$ 0 10	1 96 $\pm$ 0 02
	120	32	0 1422	77 5 $\pm$ 0 30	28 6 $\pm$ 0 26	38 6 $\pm$ 0 59	14 8 $\pm$ 0 11	19 0 $\pm$ 0 17	1 93 $\pm$ 0 02
0 1 per cent sodium fluoride	60	60	0 0866	75 7 $\pm$ 0 006	32 9 $\pm$ 0 26	43 5 $\pm$ 0 37	14 3 $\pm$ 0 07	19 0 $\pm$ 0 06	2 30 $\pm$ 0 018

\* P. e. = probable error.

510°. The ash was dissolved in a 1:1 hydrochloric acid solution and made up to 100 cc. volume. Aliquots of this solution were taken for analysis. Phosphorus was determined by the colorimetric method of Fiske and Subbarow and calcium by the method of McCrudden with 0.05 N potassium permanganate solution and a microburette. All of these analyses were made at least in triplicate and in the majority of cases were repeated in duplicate.

The results of these analyses are summarized for teeth in Table I and for bones in Table II. Due to the recognized changes in

TABLE II

*Average Composition of Bones (Tibias) of Rats Fed Basal Ration with and without Addition of Sodium Fluoride*

Dietary supplement	Length of test period	No of rats represented	Weight of dry tibia	Ash in dry tibia	Ca in dry tibia	Ca in ash	P in dry tibia	P in ash	Ca: P ratio
	days		gm.	per cent	per cent	per cent	per cent	per cent	
None (control series)	60	32	0.2769	68.2	25.6	37.9	12.1	17.8	2.09
	120	20	0.3380	70.5	26.1	37.4	12.0	17.8	2.14
0.05 per cent sodium fluoride	60	32	0.2472	68.2	25.2	37.2	12.3	18.0	2.04
	120	26	0.2929	70.0	26.9	37.3	12.3	17.5	2.14
0.1 per cent sodium fluoride	60	60	0.1568	66.2	27.2	41.03	11.9	18.0	2.28

chemical combinations, loss of CO<sub>2</sub>, etc., upon ashing bone at 510°, the results based upon the ash content of the teeth or bones are probably less reliable and significant than those based on the dry weight of unashed teeth or bones. There is no significant difference between the composition of the teeth or bones of the control animals and those given 0.05 per cent sodium fluoride in their ration. When, however, as much as 0.1 per cent of the ration is sodium fluoride, the teeth and bones were found to contain approximately 2 per cent less ash, 3 per cent more calcium, and a higher ratio of calcium to phosphorus.

## DISCUSSION

Chemical analyses, and microscopic and x-ray defraction examinations (17-20) indicate that bone is a carbonate apatite of the probable formula of  $(\text{Ca}_3(\text{PO}_4)_2)_n \cdot \text{CaCO}_3$  in which  $n = 2$  or 3.

It is logical to believe that as a result of the feeding of fluorides, the fluorine replaces the carbonate radical with the formation of a fluorapatite, the extent of the replacement varying with the concentration of fluorine in the ration. Such a simple replacement would not alter the percentage of calcium or phosphorus to any great extent and the calcium to phosphorus ratio would remain unchanged. This would explain the similarity in calcium and phosphorus content and ratios in the teeth and bones of the control animals and those receiving the lower level of sodium fluoride (0.05 per cent).

Commercial domestic types of rock phosphates have been found to contain more fluorine than corresponds to the simple fluorapatite formed as  $(\text{Ca}_3(\text{PO}_4)_2)_n \cdot \text{CaF}_2$  and Reynolds, Jacob, and Hill (21) have suggested that some of the fluorine is deposited as crystalline calcium fluoride not in combination with calcium phosphate. Rogers (22) describes fossil bone as colophane for which he gives the formula  $3\text{Ca}_3(\text{PO}_4)_2 \cdot 1-2 \text{Ca}(\text{F}_2, \text{CO}_3, \text{SO}_4, \text{O}) \cdot \text{XH}_2\text{O}$  and the fluorine content of fossil bone has been found to increase with its age probably because of a more prolonged content with fluorine-bearing waters.

The deposition of a greater amount of calcium fluoride in teeth or bones either as free calcium fluoride or in concentration with calcium phosphate would result in an increase in the percentage of calcium and a higher calcium to phosphorus ratio. It is interesting to note that the percentages of calcium, and the ratios of calcium to phosphorus in the teeth of the animals fed 0.1 per cent sodium fluoride as obtained by chemical analysis are remarkably similar to the calculated ratio of the perhaps theoretical compound colophane,  $3\text{Ca}_3(\text{PO}_4)_2 \cdot 2\text{CaF}_2$ .

## SUMMARY

Analyses have been made of the ash, calcium, and phosphorus content of the teeth and bones of fluoride-fed animals and these analyses have been compared with those of their controls which did not receive additional fluoride in their ration. When sodium

fluoride was incorporated in the ration at the level of 0.05 per cent, the analyses of teeth and bones showed no significant alterations in the percentages of ash, calcium, or phosphorus or in the calcium to phosphorus ratio.

When the concentration of sodium fluoride was increased to 0.1 per cent of the ration, the teeth and bones were lower in ash content but contained a greater percentage of calcium, lower percentage of phosphorus, with a higher calcium to phosphorus ratio.

The possible relation of these findings to the nature of the changes which affect the composition of the teeth and bones of fluoride-fed animals is discussed.

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# GRAVIMETRIC DETERMINATION OF SERUM CHOLESTEROL ADAPTED TO THE MAN AND GILDEA FATTY ACID METHOD, WITH A NOTE ON THE ESTIMATION OF LIPOID PHOSPHORUS

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Modifications of the digitonin method for the estimation of serum cholesterol and of the Fiske and Subbarow technique for estimation of serum lipid phosphorus have been combined with the Man and Gildea modification of the Stoddard and Drury method for the determination of fatty acids, in order to minimize time-consuming manipulations and the quantity of blood serum required.

Gravimetric digitonin determination of blood serum cholesterol has not been generally adopted because the technique is complicated, digitonin is expensive, and the quantitative separation of the precipitate is difficult. Colorimetric determinations of cholesterol by modifications of the Liebermann-Burchard color reaction are at times subject to inaccuracies which, according to Mühlbock and Kaufmann (18), may vary from -18 to +76 per cent. Comparative results on six sera by the Leiboff (14) cholesterol method and by the following digitonin modification differed in one instance by as much as 111 mg. per cent; in the other cases, higher values were obtained sometimes with one method, sometimes with the other. Attempts to observe the precautions recommended by Luden (15), Myers and Wardell (20), and Gardner and Williams (12) did not lessen the inconsistencies of the colorimetric method. Okey's (21) method for the determination of cholesterol by oxidation of the digitonide and Yasuda's (28) and

\* The portion of the paper dealing with phospholipids is taken from the dissertation presented by E. B. Man in partial fulfilment of the requirement for the degree of Doctor of Philosophy, Yale University, 1932.

Turner's (25) modifications of this method are subject to the same criticisms which apply to the oxidative determination of fatty acids; *i.e.*, oxidation is not specific for lipids, since contaminating materials and traces of lipid solvents alter the final results; and slight variations of temperature also affect the completeness of the reaction.

### *Cholesterol Determination*

The final solution after the determination of serum fatty acids by the Man and Gildea (16) modification of the Stoddard and Drury (24) method contains cholesterol extracted from the serum, saponified, and freed from much contaminating material. Cholesterol in an aliquot of this solution is precipitated as the digitonide. After evaporation to dryness, the cholesterol digitonide is filtered into small funnels with Jena fritted disks and weighed quantitatively. The use of these funnels facilitates microgravimetry and thus reduces the quantity and expense of digitonin.

*Apparatus*—Funnels with Jena fritted disks (Eimer and Amend Bulletin 432, No. 26032F) or funnels weighing under 10 gm. and containing a slightly larger fritted disk. A microbalance.

*Solutions*—1.0 per cent alcoholic solution of digitonin; 95 per cent alcohol redistilled from potassium hydroxide; ethyl ether redistilled with the use of an eight bulb condenser.

*Method*—After the final titration by the Man and Gildea (16) modification of the Stoddard and Drury method for the determination of serum total fatty acids, the alcoholic solution of the soaps is diluted to volume (usually 50 ml.) with redistilled 95 per cent alcohol, and an aliquot containing 0.5 to 1.5 mg. of cholesterol is taken for analysis. The solution in a 50 ml. beaker is acidified with 0.1 ml. of 0.1 *N* hydrochloric acid and, after the addition of 1 ml. of 1.0 per cent digitonin solution, is evaporated to dryness on a hot-plate at such a low heat that spattering and oxidation are avoided.

The precipitate of cholesterol digitonide is first washed with five 2 ml. portions of boiling ether which are decanted through a tared Jena funnel and is then transferred to the funnel by means of several portions of water, each of which is heated to boiling before it is filtered. It has been found advisable to use at least 10 ml. of water for the first washing, followed by five portions of 1.0 to

1.5 ml. This large initial volume facilitates solution of the excess digitonin before it is transferred to the fritted disk. If filtration proceeds with annoying slowness, agitation of the precipitate with a blunt stirring rod, or the addition of acetone to the solution in the funnel, sometimes has an accelerating effect.

The funnel and precipitate, after they have been dried in an electric oven at 95° for 45 to 60 minutes, are weighed. Washing, drying, and weighing are repeated until the weight becomes constant. The funnels are cleaned by soaking in sulfuric acid and dichromate cleaning solution. They should be reweighed, empty, before the next determination, as they sometimes lose 0.05 to 0.07 mg. between determinations.

#### *Calculation*

$$\frac{(\text{Mg. cholesterol digitonide}) (24.31)}{\text{Ml. serum in aliquot}} = \text{mg. per cent cholesterol}$$

24.31 is the factor to convert cholesterol digitonide to mg. per cent of free cholesterol.

*Remarks*—The serum to be used for cholesterol determinations should be precipitated with alcohol and ether within 2 hours of the time when the blood is taken. Several attempts to repeat determinations on serum which had been kept in the ice box a few days resulted in lower yields of cholesterol digitonide. In one instance after 8 days the cholesterol recovery was about 100 mg. per cent lower than the original determination of 547 mg. per cent, while in the same length of time the titrated fatty acids had only decreased from 30.2 to 29.3 milli-equivalents.

To determine the per cent of cholesterol extracted by refluxing blood serum with an alcohol-ether mixture for 1 hour, six 2 ml. aliquots of serum from each of two normal subjects were extracted as usual, the protein residues were combined and refluxed 3 hours with 100 ml. of redistilled 95 per cent alcohol, and, after filtration, the combined protein residue was again refluxed with 100 ml. of chloroform. The alcohol and chloroform extracts were combined and the cholesterol content determined as usual. The second extraction of the protein residues of the serum of C. W. yielded 1.27 per cent of the total cholesterol (cholesterol by first determination 175 mg. per cent; by second determination 2 mg. per cent);

from the protein residues of the serum of E. M. 1.14 per cent was recovered (cholesterol by first determination 208 mg. per cent; by the second 2 mg. per cent). Therefore the alcohol-ether extraction recovers about 99 per cent of the total cholesterol of normal serum.

There has been some difference of opinion concerning complete saponification of cholesterol esters without destruction of cholesterol (3, 5, 8, 9, 11-13, 17-19, 21, 25, 28). To determine the completeness of saponification two aliquots of the alcohol-ether extract of serum were saponified by the method employed in the Man and Gildea method for the determination of serum fatty acids (heated with 0.1 ml. of a solution made by dissolving 50 gm. of potassium hydroxide in 50 ml. of water, for 45 to 60 minutes until dry, the terminal heating being conducted under nitrogen) and two aliquots were heated with 0.2 ml. of potassium hydroxide solution for at least 20 hours under a reflux condenser before the final evaporation to dryness. The cholesterol contents of two different sera, one from a normal subject and the other from a diabetic patient, determined after both long and short saponification agreed within the experimental error of the method: in the serum of the normal subject after short saponification the duplicates were 190 and 190, after long saponification 186 and 175 mg. per cent; in the serum of a diabetic after short saponification 236 and 216, after long saponification 230 and 222 mg. per cent. It has been found that if at the end of the process of saponification, heating is continued after complete evaporation of the alcohol, lower cholesterol values are obtained.

The temperature of the oven in which the cholesterol digitonide is dried must be carefully regulated. On one occasion, through a fault in the electrical connections, the temperature of the oven increased. Quantitative recoveries of cholesterol were obtained at 108°, but, when the temperature rose to 115°, drying for an hour turned the precipitate yellow and the weight of the cholesterol digitonide was far below the theoretical level.

Reports vary as to the amount of cholesterol which is precipitated quantitatively by the use of 1.0 ml. of 1.0 per cent digitonin solution. Mühlbock and Kaufmann (18) stated that for 10 mg. of cholesterol the optimum quantity of digitonin is 5 ml. of 1.0 per cent solution, while an increase in the quantity of digitonin solution magnified the weight of the precipitate. Turner (25),

## E. B. Man and J. P. Peters

in her modification of Okey's method, recommended 0.1 ml. of 1.0 per cent digitonin for 0.5 to 1.5 mg. of cholesterol. Gardner and Williams (12) used 1.5 to 2.5 per cent of digitonin in excess of the theoretical combining quantity for complete precipitation of cholesterol digitonide. To investigate this point different quantities of cholesterol solution were precipitated as cholesterol digitonide and weighed. The results which are given in duplicate

TABLE I  
*Precipitation of Cholesterol by Digitonin*

Cholesterol taken	Cholesterol digitonide	Cholesterol recovered
<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
0 445	1.89	103.2
	1.74	95.2
1 120	4.77	103.5
	4.75	103.0
1 080	4.34	97.5
	4.30	96.8
1 558	6.25	97.5
	6 26	97.7

TABLE II  
*Recovery of Cholesterol from Serum*

Volume of serum	Cholesterol in serum	Cholesterol in solution added to serum	Added cholesterol recovered
<i>ml.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
1.6*	1.58	1 12	98.9
			99.3
0 2	0.43	0 50	95.2
			96.0

\* 2 ml. of 1.0 per cent digitonin solution were used.

in Table I show that 1.0 ml. of 1.0 per cent digitonin permits the quantitative precipitation of at least 0.5 to 1.5 mg. of cholesterol.

To test the method further, the cholesterol content of serum and of serum plus an alcohol-ether solution of cholesterol has been determined. In each instance, no step in the technique was omitted. Theoretical recoveries of the added cholesterol were achieved, as can be seen from the data in Table II.

Since the funnels lose weight when used (occasionally 0.07 mg. between successive determinations) and since the precipitate may lose a few hundredths of a mg. each time it is dried, it has been impossible to obtain duplicate weights of cholesterol digitonide which agree much more closely than 0.22 mg. If the aliquot contains only 0.5 mg. of cholesterol, the precipitate of cholesterol digitonide weighs approximately 2.05 mg., and the error is about 10 per cent. The weight of the precipitate varies greatly, depending on the size of the aliquot in relation to the concentration of cholesterol in the serum. With this method the serum cholesterol of ten normal subjects, in the postabsorptive state, ranged from 162 to 256 mg. per cent, with an average value of 208 mg. per cent.

This modification has been used for more than 156 determinations of serum cholesterol. Of these, ten observations have been rejected because the duplicates varied by more than 10 per cent and it was impossible to attempt a third determination because the serum was no longer fresh. In 146 accepted observations the average difference between duplicates was 3.7 per cent; the mean error of a single observation calculated by the method of least squares was 5.5 mg. of cholesterol per 100 ml. of serum.

#### *Lipoid Phosphorus Determination*

Serum phosphatides have been determined either by estimation of lipid phosphorus or, after acetone precipitation of the phospholipids, by oxidation with sulfuric and chromic acids and subsequent iodometric titration of the excess chromic acid (4). The latter has proved rather unsatisfactory because of the difficulty of separating quantitatively phosphatides from other fats and because of uncertainty as to the oxidative factor applicable to all phospholipids (6, 23, 26). The small quantity of blood serum available from pathological subjects makes impracticable gravimetric determination of lipid phosphorus. The colorimetric method of Fiske and Subbarow (10) for the estimation of lipid phosphorus is less sensitive to slight variations in acid content of the solutions and to interfering substances, and is quicker than Briggs' modifications (7) of the Bell and Doisy (1) technique (22).

In the following modification of the Fiske and Subbarow method, an aliquot of the same alcohol-ether extract used for the determination of serum fatty acids is evaporated to dryness. The

lipoid phosphorus is hydrolyzed, and the resulting inorganic phosphate determined colorimetrically by the addition of ammonium molybdate and the reducing agent, 1, 2, 4-aminonaphtholsulfonic acid.

*Reagents*—10 N sulfuric acid. Molybdate Solution III and the standard phosphate solution, prepared as directed by Fiske and Subbarow (10). 1, 2, 4-aminonaphtholsulfonic acid purified in accordance with the directions of the same authors, but dissolved in freshly prepared solutions of sodium bisulfite and sodium sulfite. Superoxol and concentrated nitric acid.

*Method*—8 ml. of the alcohol-ether extract of serum (equivalent to 0.32 ml. of serum) prepared for the determination of serum fatty acids, as described by Man and Gildea (16), are measured into a Pyrex test-tube 15 mm. in diameter and at least 145 mm. long. Two glass beads are added. The test-tube is placed in a hot water bath at about 30° and is heated cautiously (preferably on a hot-plate at low heat) for 4 to 6 hours, until all alcohol and ether have evaporated.

0.5 ml. of 10 N sulfuric acid is added and the tube is heated cautiously over a microburner with a flame 1 cm. in height. As soon as the digestion mixture becomes charred, 2 drops of concentrated nitric acid are added, followed by 1 more drop of nitric acid after the mixture becomes yellow. When the digestion is almost complete, 0.1 ml. of superoxol is added. Heating is continued just long enough to render the mixture colorless and to drive off any excess superoxol. During the digestion the tube is rotated and tipped to remove any organic matter which may have adhered to the upper part of the test-tube during the evaporation of the alcohol and ether.

About 6 ml. of distilled water, 1 ml. of molybdate solution, and 0.4 ml. of the reducing agent are added to each tube after the digestion and the whole is diluted to 10 ml. The color is matched in a colorimeter after the solution has been allowed to stand about 5 minutes. If the equivalent of only 0.16 ml. of serum is used, the volume and all reagents are halved. If the color which develops is intensely blue, 0.5 ml. of 10 N sulfuric acid, 1 ml. of molybdate solution, and 0.4 ml. of sulfonic acid are added and the total volume made up to 20 ml. before the colors are compared in the colorimeter, because the maximum quantity of phosphorus which can be determined with dilution to 10 ml. is only 0.06 mg.



The standard is prepared by adding to 5 ml. of the phosphate standard in a 100 ml. volumetric flask about 60 ml. of water, 5 ml. of 10 N sulfuric acid, 10 ml. of the molybdate solution, and 4 ml. of the aminonaphtholsulfonic acid. This is then diluted to volume and the color allowed to develop for about 5 minutes.

For a blank determination 8 ml. of the alcohol-ether mixture are evaporated to dryness in a large Pyrex test-tube. 5 ml. of the phosphate standard are placed in the tube and are digested with 5 ml. of 10 N sulfuric acid, 30 drops of concentrated nitric acid, and 1 ml. of superoxol. After the water has evaporated, the mixture is heated for the same length of time usually required for the digestion of phospholipids (a total of about 25 minutes for evaporation and digestion), and is then transferred quantitatively to a 100 ml. volumetric flask. 60 ml. of water, 10 ml. of molybdate solution, and 4 ml. of aminonaphtholsulfonic acid are added. After dilution to volume, the blank is compared with the standard in a colorimeter.

#### Calculations

$$\text{I. } \frac{0.04 S}{U} = \text{mg. phosphorus in blank and unknown (when the unknown has been diluted to 10 ml.)}$$

$$\text{II. } \frac{0.04 S}{B} = 0.04 \text{ mg. phosphorus} + \text{phosphorus in blank in 10 ml. solution}$$

$$\text{III. } \frac{I - (II - 0.04)}{\text{Volume of serum used}} \times 1000 = \text{mg. lipid P in 1000 ml. serum}$$

$S$  = reading of standard;  $U$  = reading of unknown;  $B$  = reading of blank.

The usual volume of serum =  $(8/50) \times 2 = 0.32$  ml.

*Remarks*—Inorganic phosphorus and lecithin phosphorus, the latter in simple solution and when added to serum, have been recovered quantitatively by this method with a maximum error of  $\pm 5.0$  per cent (see Table III). That inorganic phosphorus does not contaminate the alcohol-ether solution of serum lipids was demonstrated by two experiments, confirming the observations of Bloor (2). The sum of serum lipid phosphorus, 10.70 mg. per cent, and of acid-soluble phosphorus, 4.37 mg. per cent, agreed within the experimental error with the serum total phosphorus of

TABLE III  
*Recovery of Lecithin Phosphorus*

	Total P*	Final dilution of solution for lecithin P determination	P recovered
	<i>mg.</i>	<i>ml.</i>	<i>per cent</i>
Lecithin solutions in alcohol-ether	0 0478	10 0	98 4
	0 0547	10 0	97 6
	0 082	20 0	100 0
Serum plus lecithin solution	0 0336 Serum		
	0 1308 Lecithin	50 0	98 0
	0 0354 Serum		
	0 1268 Lecithin	50 0	97 0
	0 0289 Serum		
	0 2493 Lecithin	100 0	95 0

\* Total P was calculated by multiplying the weight of lecithin used by the factor 0.0385, the derivation of which was discussed by Man and Gildea (16).

TABLE IV  
*Serum Lipids of Normal Subjects in Postabsorptive State*

Name	Total fatty acids	Lipoid P	Cholesterol	Name	Total fatty acids	Lipoid P	Cholesterol
	<i>m -eq</i>	<i>mg per cent</i>	<i>mg per cent</i>		<i>m -eq</i>	<i>mg per cent</i>	<i>mg. per cent</i>
P. H.	14 3	9 8	215	C. R.	17 4	10 4	212
E. M.	13 2	9 9	207		13 7	9 7	216
	12 7	8 9	223	B. B.	16 1	10 7	248
	13 4	9 0		T. K.	10 8	8 4	168
	14 9	9 5		R. B.	14 8	10 1	
C. W.	12 3	8 6	178	V. M.	16 0	10 1	
	10 4	7 1		E. G.	13 4	8 7	
J. B.	15 8	11 3	256	H. D.	10 2	8 1	
J. H.	12 6	9 0	205	H. H.	10.2	7 8	
L. B.	10 4	6 9	162	E. C.	12 3	7 8	
P. L.	13 2	8 4	199	B. G.	13 0	9 0	
				M. C.	12 9	8 1	
				M. H.	13 3	7 8	

14.59 mg. per cent. The addition of sulfuric acid, molybdate solution, and aminonaphtholsulfonic acid to an evaporated aliquot of alcohol-ether extract produced no color, although it requires

less than 0.003 mg. of P in 10 ml. of solution to give a blue color. The completeness of extraction of phospholipids by refluxing 2 ml. of serum with 25 ml. of alcohol-ether solution (3 parts of alcohol to 1 of ether) and then filtering the protein residue from the lipid extract was demonstrated in two different experiments by reextracting the protein residues and filter paper from (1) eight aliquots, each of 2 ml. of the same serum, and (2) four aliquots of another serum. Reextraction was accomplished by refluxing 2 hours with 95 per cent alcohol, and after filtration of the alcohol, refluxing 1 hour with ethyl ether. In these two experiments these prolonged extractions with alcohol and ether recovered 1.52 and 1.38 per cent of the lipid phosphorus. A third reextraction of the combined protein residues from the two previous experiments gave less than 0.3 per cent of the total lipid phosphorus. These figures show that the original alcohol-ether extraction of serum yields at least 98 per cent of the total lipid phosphorus.

Twenty-four determinations of lipid phosphorus on nineteen normal subjects in the postabsorptive state varied from 6.9 to 11.3 mg. per cent, with an average of 8.95 mg. per cent of phosphorus (see Table IV).

#### SUMMARY

A modification of the digitonin method for the determination of serum cholesterol has been adapted to the analysis of the solution remaining after the determination of serum fatty acids by the Man and Gildea modification of the Stoddard and Drury technique. The Fiske and Subbarow method has been adapted to the estimation of lipid phosphorus in an aliquot of the alcohol-ether extract of serum prepared for the determination of fatty acids.

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## TRIANHYDROPERIPILOGENIN

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In the course of our previously reported examination of the glucosides of *Strophanthus eminii*<sup>1</sup> it was found that the relatively stable glucoside fraction, when subjected to the vigorous conditions necessary for hydrolytic cleavage, gave a trianhydroaglucone,  $C_{23}H_{28}O_2$ , which was obviously formed by loss of 3 mols of water from an aglucone,  $C_{23}H_{34}O_5$ . Since periplogenin was isolated by cleavage of the easily hydrolyzable glucoside fraction, it was first believed that the above substance was a trianhydroperiplogenin. In order to check this view, periplogenin itself was subjected to similar severe treatment; i.e., heating at 100° with 5 per cent methyl alcoholic hydrochloric acid. A trianhydroperiplogenin resulted in rather poor yield, which proved to be not identical but isomeric with the above trianhydroaglucone from *Strophanthus eminii*.

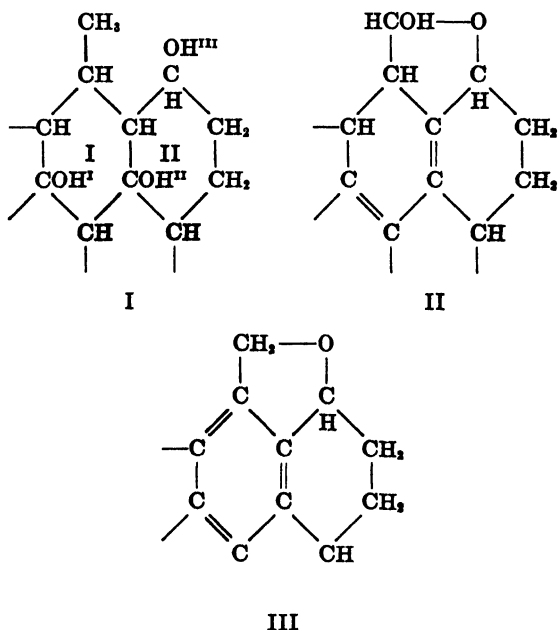
Thus, all of the hydroxyl groups of periplogenin, including the secondary hydroxyl group ( $OH^{III}$ ) of Ring II, were removed. In the case of no other related aglucone has evidence been obtained of the removal of this secondary hydroxyl group under similar conditions. Digitoxigenin and gitoxigenin give respectively a monoanhydro derivative and a dianhydro derivative (digitaligenin), both of which still possess the acylatable secondary hydroxyl ( $OH^{III}$ ). Therefore, in periplogenin (Formula I) the removal of this additional hydroxyl group must be induced by a series of steps similar to those already noted in the case of the dehydration of strophanthidin.<sup>2</sup> Although the intermediate substances were not isolated in the case of periplogenin, the most

<sup>1</sup> Jacobs, W. A., and Bigelow, N. M., *J. Biol. Chem.*, **99**, 521 (1932-33).

<sup>2</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **59**, 713 (1924).

probable picture of what happens is as follows. First,  $\text{OH}^{\text{I}}$  is removed and the new double bond in the resulting monoanhydro derivative labilizes  $\text{OH}^{\text{II}}$  with consequent formation of a dianhydro derivative. The position occupied by one of these double bonds, perhaps after preliminary rearrangement, then labilizes  $\text{OH}^{\text{III}}$ , with the formation of a trianhydroperiplogenin. The last step here differs from what has been noted in the case of the closely related strophanthidin,<sup>3</sup> for in the latter the third hydroxyl group to be removed is not the secondary hydroxyl ( $\text{OH}^{\text{III}}$ ) but the lactol hydroxyl of dianhydrostrophanthidin (Formula II) in which the secondary hydroxyl ( $\text{OH}^{\text{III}}$ ) is involved in the oxidic bridge.

Since in the case of trianhydrostrophanthidin a benzenoid structure (Formula III) has been indicated by its behavior on



hydrogenation and oxidation,<sup>4</sup> we have attempted to check the assumed analogous structure of trianhydroperiplogenin by a

<sup>3</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **63**, 123 (1925).

<sup>4</sup> Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 805 (1927).

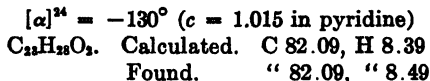
similar study. However, a difference has been noted. Whereas it has not been possible to hydrogenate the double bonds of trianhydrostrophanthidin, with the exception of the original one contained in the unsaturated lactone side chain, trianhydroperiplogenin was readily hydrogenated. In acetic acid solution with Adams and Shriner's platonic oxide catalyst, 4 mols of hydrogen were absorbed with the formation of a mixture of isomers of octahydrotrianhydroperiplogenin, from which one was isolated in pure form. This substance was apparently isomeric with the saturated desoxy lactones obtained by Windaus and coworkers from gitoxigenin, digitaligenin, and digitoxigenin.

On attempting to oxidize trianhydroperiplogenin with permanganate in acetone solution by the method which caused degradation of the  $\Delta^{\beta}, \gamma$ -lactone side chain of trianhydrostrophanthidin<sup>4</sup> to a carboxyl group, although oxidation occurred, no tangible product could be isolated.

It is difficult to know whether the benzenoid behavior of trianhydrostrophanthidin is partly supported by a stabilizing influence of the oxidic bridge and the divergent behavior of trianhydroperiplogenin is caused by its less stable configuration, or whether in the latter substance the double bonds are not contained in one benzenoid ring. It is hoped to determine this point more definitely at a later date by ultra-violet absorption spectra measurements.

#### EXPERIMENTAL

*Trianhydroperiplogenin*.—A solution of 0.5 gm. of periplogenin in 5 cc. of absolute methyl alcohol containing 5 per cent of dry hydrogen chloride was heated in a sealed tube at 100° for 15 minutes. On cooling the solution deposited the crystalline reaction product. Recrystallization gave 0.12 gm. of fine, light yellow needles which melted at 191–193°, depending on the rate of heating. When covered with a drop of sulfuric acid, the substance gave a deep garnet-red solution which became gradually ringed with a bright purple and finally changed to blue. The Legal test was positive.





*Octahydrotrianhydroperiplogenin*—A solution of 0.2 gm. of trianhydroperiplogenin in acetic acid was hydrogenated with the platinic oxide catalyst of Adams and Shriner. 4 mols of hydrogen were absorbed within 3 hours. Concentration of the filtered solution gave a colorless syrup which crystallized on addition of a little alcohol. 90 mg. of feathery needles were obtained. Five recrystallizations were found necessary to separate the substance from stereoisomers. The pure substance crystallized in the form of fine interlocking needles which melted at 176–177°. Its solution in sulfuric acid was colorless.  $[\alpha]_D^{25} = +16.4^\circ$  ( $c = 0.52$  in chloroform). For analysis the substance was dried at 115° and 15 mm.

$C_{22}H_{34}O_2$ .	Calculated.	C 80.17, H 10.54
	Found.	" 80.45, " 10.34

With the amount of material at our disposal no attempt was made to fractionate out other isomeric substances which were also formed.

Trianhydroperiplogenin in ethyl acetate likewise absorbed 4 mols of hydrogen, but much more slowly than in acetic acid solution.

# THE CAROTENE CONTENT, VITAMIN A POTENCY, AND ANTIOXIDANTS OF BUTTER FAT

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The association of color with vitamin A activity was first reported by Palmer (1) and Steenbock and associates (2, 3). A number of investigators have shown that destruction of color in butter is accompanied by a loss of vitamin A activity (4-7). However, the experiments of Stephenson (8) indicated that decolorization of butter brought about by treatment with charcoal did not impair its vitamin A potency. Holmes, Lava, Delfs, and Cassidy (9) demonstrated that carotene and vitamin A were adsorbed by porous alumina from petroleum ether solutions. In view of the work of Moore (10) and Capper (11), which showed that carotene can serve as a source of vitamin A for rats, it appeared that the removal of the color (carotene) from butter in itself would reduce the vitamin A activity.

*Determination of Carotene Content of Butter Fat*—Solutions of carotene in petroleum ether, butter fat, and butter fat to which varying amounts of carotene had been added were examined for carotene content by the colorimetric and spectrophotometric methods.

The colorimetric estimations were made in this laboratory with a Leitz colorimeter at a temperature of 40° according to the method of Willstätter and Stoll as described by Palmer (12).

The spectrophotometric determinations were made according to the procedure of Schertz (13).<sup>1</sup> Solutions to be examined were transferred to glass-stoppered bottles and sent by express to the

<sup>1</sup> The authors are indebted to the Bureau of Standards of the United States Department of Commerce for the determination of the transmittancy of the solutions.

Bureau of Standards where they were usually examined within 48 hours after arrival. In the case of the butter fats about 7 gm.

TABLE I

*Comparative Values of Carotene\* Content of Petroleum Ether Solutions and of Butter Fat†*

The values for carotene are expressed as mg per 100 cc in the case of petroleum ether solutions and in mg per 100 gm. for butter fat.

Solution and butter fat sample No	Material examined	— Log <sub>e</sub> transmittancy for 2 cm	Carotene content		Theoretical carotene	Carotene added	Carotene recovered		Recovery	
			Colorimeter	Spectrophotometer			Colorimeter	Spectrophotometer	Colorimeter	Spectrophotometer
			mg	mg	mg	mg	mg	mg	per cent	per cent
1	Carotene in petroleum ether		1 94		2 04	2 04	1 94		95 1	
3	“ “		2 05		2 04	2 04	2 05		100 4	
4	“ “	0 83	2 03	2 08	2 02	2 02	2 03	2 08	100 4	102 9
5	Butter fat, fresh		1 98			0 00				
6	Sample 5 1 wk. later	0 13	1 87	0 36		0 00				
7	Sample 5 + carotene, fresh		5 71			2 0				
8	Sample 5 + carotene, 1 wk. later	0 65	6 25	2 30	2 36	2 0	4 06	1 94	203 0	97 0
9	Butter fat	0 16	1 66	0 57		0 00				
10	Sample 9 + carotene	0 27	2 33	0 97	0 98	0 408	0 67	0 40	164 2	98 1
11	“ “	0 41	3 84	1 50	1 59	1 02	2 18	0 93	213 7	91 2
12	“ “	0 65	5 54	2 47	2 61	2 04	3 88	1 90	190 2	93 1
13	“ “	0 95	8 21	3 53	3 63	3 06	6 55	2 96	214 0	96 4
14	“ “	1 21	10 31	4 50	4 65	4 08	8 65	3 93	212 0	96 3

\* Carotene supplied by Dr. F M Schertz was used in preparation of Samples 1, 7, 8, 10-14 and a sample obtained from The British Drug Houses Ltd. was used in the preparation of Samples 3 and 4

† The — log<sub>10</sub> transmittancy for 2 cm for decolorized butter fat Sample 5 was 0 068; for decolorized butter fat Sample 9, 0 035

were weighed out and transferred to a glass-stoppered bottle. About 50 cc. of petroleum ether were added. Before examination

this solution was made up quantitatively to 100 cc. volume with petroleum ether. A sample of butter fat which had been decolorized was treated in the same way and a correction was made in the final calculation for the transmittancy of this material. Colorimetric examination showed that there was no appreciable loss in color of the butter solutions between the time they were prepared in this laboratory and when they were examined spectrophotometrically (butter Samples 7 and 8, Table I).

Table I gives the results of the colorimetric and spectrophotometric estimation of carotene in the different materials.

The results show that carotene in petroleum ether can be estimated with a fair degree of accuracy by the colorimeter. The low result obtained with Solution 1, in which less than the theoretical amount of carotene was found, may be due to impurities in the crystalline carotene. This is substantiated by the results obtained with butter fat Samples 10 to 14 inclusive. Petroleum ether carotene Solution 1 was used in the preparation of these butter samples and the carotene found (spectrophotometrically) was uniformly less than 100 per cent. In the case of petroleum ether Solution 4 the carotene found by either the colorimetric or spectrophotometric method agreed closely with the calculated value.

The original carotene content of butter Sample 5 determined colorimetrically was about 5 times higher than the value found spectrophotometrically. Goldblatt and Barnett (14) state that, "the color intensity of carotene in oil is several times that of an equal quantity dissolved in petroleum ether. . . ." When a known amount of carotene was added to this sample, the theoretical amount was obtained spectrophotometrically, while more than twice that amount was determined colorimetrically.

Quantities of carotene varying from 0.408 to 4.08 mg. were added to 100 gm. of butter and the carotene determined colorimetrically and spectrophotometrically. In all cases the amount found by the colorimetric method was from 164.2 to 214 per cent and by the spectrophotometric method from 91.2 to 98.1 per cent. As stated earlier the low results may have been due to impure carotene, since the colorimetric examination of its petroleum ether solution gave values lower than the theoretical amount.

A recalculation of these values on the basis of the amount of carotene shown by the colorimetric analysis of the petroleum

ether solution shows 103.0, 95.9, 97.9, 101.7, and 101.2 per cent of the calculated values by the spectrophotometric method for the butter Samples 10, 11, 12, 13, and 14 respectively.

Since Palmer (1) has shown that butter contains small amounts of xanthophyll and since xanthophyll shows absorption at 435.8  $m\mu$ , the amount of carotene shown in the original butter samples as determined by the spectrophotometric method may be slightly high.

*Effect of Charcoal Treatment on Vitamin A Content of Butter Fat*—1 part of melted butter fat, at a temperature of about 40° was dissolved in 2 parts of petroleum ether. To 750 cc. of the mixture 30 gm. of charcoal<sup>2</sup> were added with shaking. After filtering

TABLE II  
*Decolorization of Butter Fat with Charcoal (10 Cc. of Butter Fat Plus 10 Cc. of Petroleum Ether)*

Charcoal used	Color of butter fat after treatment	Charcoal used	Color of butter fat after treatment
<i>gm.</i>		<i>gm.</i>	
0 10	Dark yellow	0 57	Slight trace yellow
0 20	Light "	0 58	Questionable trace yellow
0 40	Trace "	0 59	Colorless
0 50	" "	0 60	"
0 55	" "	0 80	"
0 56	" "	1 00	"

through a Buchner funnel the filtrate was made up to the original volume with petroleum ether and the charcoal treatment repeated. Upon evaporation of the petroleum ether a lard-white product was obtained. The amount of charcoal used was in excess of that actually required to remove the color of butter fat as shown from experiments recorded in Table II.

A sample treated as above was fed to rats in amounts of 100, 300, and 1000 mg. daily.

Young albino rats were used as test animals. The curative method was employed for the determination of vitamin A. The basal vitamin A-free diet was composed of casein<sup>3</sup> 18, dextrin 61,

<sup>2</sup> "Special nuchar," Industrial Chemical Sales Company, Inc., New York.

<sup>3</sup> The casein was extracted continuously for 72 hours each with ether, alcohol, and acidulated water.

yeast 6, salt mixture (15) 4, powdered agar-agar 3, and lard 8. The animals were irradiated for 15 minutes three times a week with ultra-violet light. The supplements were weighed out on the torsion balance and fed to the animals in small glass casters.

The results given in Table III show that no appreciable amount of vitamin A remained in the butter fat after treatment with charcoal. Although the survival period of rats on these samples was not unlike that of the negative controls which received no

TABLE III  
*Vitamin A Activity of Butter Products Fed to Rats. Experimental Period 56 Days*

Butter sample	Daily dose	Average initial weight*	Average final weight	Average gain	Condition at end of experiment	No of animals
	mg	gm	gm	gm		
No treatment	0 0	70 5	62 4	-8 1	Dead	8
Untreated butter	100	60 5	141 0	+75 5	Good	2
Decolorized with charcoal	100	62 1	49 4	-12 7	Dead	7
“ “	300	58 3	35 7	-22 6	“	3
“ “	1000	45 0	36 5	-8 5	“	2
Barely decolorized with charcoal	100	51 0	44 0	-7 0	“	2
Light colored butter	100	72 7	133 3	+60 6	Ophthalmia	4
	125	52 5	109 1	+56 6	Good	6
Medium “ “	60	81 0	73 5	+11 7†	Ophthalmia	4
	70	72 6	152 3	+79 0	Good	6
Dark “ “	30	72 5	88 5	+16 0	Ophthalmia	4
	40	76 2	117 2	+41 0	Good	4

\* At end of depletion period

† Made an average gain of 19.2 gm., then lost weight.

supplement, their behavior was different. There was a rapid loss in weight followed by a period in which the weight remained stationary for a few days, followed by further rapid decline and death. This would indicate that the decolorized butter fat contained small amounts of vitamin A.

If the vitamin A content of butter is due both to its carotene content and to preformed vitamin A, it appeared that butter which had been treated with an amount of charcoal barely sufficient to remove the color might have vitamin A activity. Feeding

such butter in 100 mg. daily doses (Table III) failed to reveal any appreciable activity.

*Carotene and Vitamin A Activity*—Since carotene may function as vitamin A it seemed desirable to attempt to determine what part of this activity was due to carotene. Three samples of butter fat were used in this experiment. The results of the analyses are reproduced in Table IV together with other related data.

The results of the feeding trials are presented in Table III. The data show that these three butter samples with different carotene contents had different vitamin A activities, although these factors

TABLE IV  
*Carotene Analyses of Butter Samples*

- Log <sub>10</sub> trans- mittancy for 2 cm *	Physical appearance	Carotene per 100 gm butter Calcu- lated	Daily dose required to protect	Carotene in daily dose	Activity due to carotene		
		mg	mg	mg	per cent	per cent	per cent
0 347	Deep yellow	1 21	40	0 000484	9 7†	24 0‡	96 8§
0 200	Yellow	0 793	70	0 000555	11 1†	27 7‡	111 0§
0 035	Very light yellow	0 143	125	0 000179	3 4†	8 9‡	35 8§

\* Determined by the United States Department of Commerce, Washington.

† Calculated on the assumption that 0 005 mg. of carotene will supply the vitamin A requirements of young rats

‡ Calculated on the assumption that 0 002 mg. of carotene will supply the vitamin A requirements of young rats.

§ Calculated on the assumption that 0 0005 mg. of carotene will supply the vitamin A requirements of young rats.

may not be correlated in all samples of butter. Wilbur, Hilton, and Hauge (16) have presented evidence which indicates that color is not a reliable measure of the vitamin A potency of butter, although the yellow pigment, carotene, contributes to the vitamin A activity of butter.

The per cent of vitamin A activity of the butter samples that could be attributed to carotene was 9.7, 11.1, and 3.4. These figures were calculated on the assumption that 0.005 mg. of carotene is required to produce limited growth of the rat. It appears from experiments not yet completed that considerably less than

this amount of carotene fed in vitamin A-free butter fat is sufficient to maintain young rats at a low rate of gain. Recently Goldblatt and Barnett (14) have demonstrated that 0.0005 mg. of carotene in Wesson oil is equivalent to 1 Sherman unit of vitamin A. If it should prove that this small amount is completely utilized from butter fat then approximately all of the vitamin A activity of the two darker samples and 35 per cent of the activity of the light colored sample could be attributed to their carotene content (Table IV). At this time it would seem safe to conclude that an appreci-

TABLE V  
*Fading of Carotene in Butter Fats after Treatment with Charcoal. Mg. of Carotene per 100 Gm. of Fat (Colorimeter)*

Butter sample	0 hr.	1 hr., 30 min.	2 hrs., 30 min.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.
Charcoal-decolorized + carotene	5.09	4.53	4.15	1.5	0.64	0.56	0.16	0.08
“ “	5.36		1.5	0.54	0.37	0.23	0.12	0.05
Untreated	1.70			1.76	1.71			1.71
“ + carotene	4.55			4.82	4.82			4.65
Charcoal-decolorized + carotene	2.46			0.96	0.69			0.60
Untreated + decolorized	0.59			0.59	0.55			0.59
Untreated + decolorized + carotene	3.05			3.66	2.95			2.78
Decolorized + carotene + hydroquinone	2.74			2.73	2.84			2.90

able amount of the vitamin A activity of butter may be due to its carotene content.

*Antioxidants of Butter Fat*—In connection with the investigation of the accuracy of the colorimetric method for the determination of carotene in butter fat it was found that carotene added to decolorized butter fat faded rapidly. The color of natural butter however is quite stable. Banks and Hilditch (17) reported that the natural antioxygens present in olive and linseed oils could be removed by boiling with water. Baumann and Steenbock (5) p. 77) found evidence that indicates the presence of a material in crude carotene extracts which protects the carotene from destruction by ultra-violet radiations. Cady and Luck (18) found that



treatment of cod liver oil with  $\text{SO}_2$  destroyed its vitamin A potency in 15 minutes, while similar treatment of butter did not appreciably affect its potency after 2 hours. They suggested that the vitamin A of butter was protected by naturally occurring substances. Marcus (19) found that the vitamin A of cod liver oil concentrates was destroyed when the material was deposited on reduced nuchar and believed the nuchar acted as an oxidative catalyst. He also reported that the destruction was inhibited by hydroquinone. Mattill (20) reported that phenols have antioxygenic capacity because of the position of their hydroxyl groups. When the hydroxyl groups are in the ortho and para positions they are active; when in the meta position they are inactive.

Table V gives the results of experiments which demonstrate the presence of substances in butter fat which protect the carotene from destruction. The determinations of carotene were made colorimetrically. It is recognized that the values are too high because of inaccuracies in the colorimetric determination applied to butter fat. Nevertheless, they serve here to show relative fading of the samples examined.

The data show that when carotene is added to butter fat which has been decolorized with charcoal, rapid fading of the pigment occurs. Practically all the carotene has disappeared at the end of 120 hours. Untreated butter fat does not fade under similar conditions. Moreover carotene added to untreated butter fat is stable. Apparently butter contains protective substances which are removed or destroyed by treatment with charcoal. Relatively small amounts of hydroquinone added to charcoal-decolorized butter fat prevent the destruction of the color of butter as do the naturally occurring protective substances. When carotene is added to an equal quantity of charcoal-decolorized butter fat and untreated butter fat, no fading takes place. It appears from these experiments that the substances which occur in natural butter and which protect the carotene of butter from destruction act as antioxidants.

#### SUMMARY AND CONCLUSIONS

1. The estimation of the carotene content of melted butter by direct comparison with a dichromate standard in a colorimeter is unreliable. The results obtained are several times too high.

Carotene dissolved in butter fat shows a color intensity several times higher than when dissolved in petroleum ether.

Carotene in petroleum ether can be estimated colorimetrically with a good degree of accuracy.

2. Carotene in petroleum ether and in butter fat dissolved in petroleum ether can be determined with considerable accuracy by means of the spectrophotometric method.

3. Treatment of butter fat with charcoal destroys its color and vitamin A activity.

4. The carotene of butter may account for an appreciable amount of its vitamin A activity.

5. Butter fat contains natural antioxidants which protect the carotene from oxidation. The antioxidants of butter fat are destroyed or removed by treatment with charcoal.

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## ON A DIPEPTIDE PHOSPHORIC ACID ISOLATED FROM CASEIN

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The object of the present investigation was 2-fold. It aimed at the discovery of first, the amino acid, or the amino acids, of the molecule of casein to which the phosphoric acid residues are attached, and second, of the amino acids which are attached to those containing the phosphoric acid residue. From the earlier work of Bunge,<sup>1</sup> Levene and Alsberg,<sup>2</sup> Salkowski,<sup>3</sup> and others, and particularly from the more recent work of the two Posternaks<sup>4</sup> and of Rimington,<sup>5</sup> it is now known that it is possible to isolate from the phosphoproteins fragments of comparatively small size still containing the phosphoric acid residue. Recently, Lipmann and Levene<sup>6</sup> isolated serinephosphoric acid from the products of hydrolysis of vitellinic acid. The study of these simpler phosphopeptides seemed to us of promise for the discovery of structural differences between individual phosphoproteins. Early this year our work was extended to casein. In an article by Levene and Schormüller<sup>7</sup> on the synthesis of tyrosinephosphoric acid, it was mentioned that from the casein phosphopeptone on hydrolysis with hydrochloric acid a substance was obtained with the ratio of N:P = 1.2. The yield of this substance was very small; a sub-

\* Commonwealth Fund Fellow.

<sup>1</sup> Bunge, G., *Z. physiol. Chem.*, **9**, 49 (1885).

<sup>2</sup> Levene, P. A., and Alsberg, C. L., *J. Biol. Chem.*, **2**, 127 (1906-07).

<sup>3</sup> Salkowski, E., *Z. physiol. Chem.*, **32**, 245 (1901).

<sup>4</sup> Posternak, S., *Compt. rend. Acad.*, **184**, 306 (1927); **186**, 1762 (1928).  
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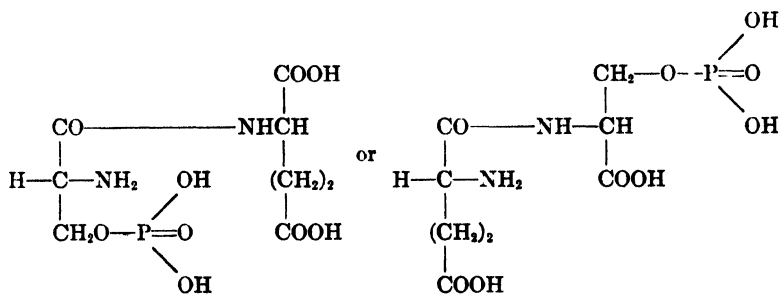
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<sup>6</sup> Lipmann, F. A., and Levene, P. A., *J. Biol. Chem.*, **98**, 109 (1932).

<sup>7</sup> Levene, P. A., and Schormüller, A., *J. Biol. Chem.*, **100**, 583 (1933).

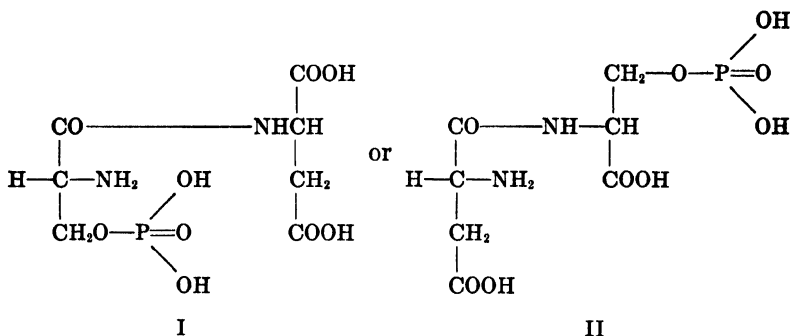
# CORRECTION

On page 712, Vol. 101, No. 3, August, 1933, the structural formulæ should read





stance with the N:P ratio of 1.3 to 1.5 was obtained in much larger proportions. It was evident that the material consisted of a mixture of phosphorylated amino acids with amino acids or with phosphorylated di- or polypeptides. The methods previously employed for the separation of the individual substances consisted in fractional precipitation of their barium salts by means of alcohol, a method very troublesome and as can be seen from the experimental part, not entirely satisfactory. It was therefore concluded to attempt the separation by fractional crystallization of the brucine salt. This procedure proved successful and led to the isolation of a crystalline brucine salt of a phosphorylated dipeptide of constant composition and constant melting point. The dipeptide consisted of serine and glutamic acid. As yet it is not known which of the two possible structures (I or II) is to be assigned to the phosphorylated dipeptide.



While this work was in progress there appeared a note by Lipmann<sup>8</sup> on the isolation from the products of hydrolysis of casein of serinephosphoric acid in the form of its barium and silver salts and when the work was ready for publication, a note in the proceedings of The Fourteenth International Physiological Congress by G. Schmidt on the isolation of a substance identical with our own came to our notice. The communication contained no details of the method of preparation, nor any analytical data. We publish our results particularly because of the method developed by us for the separation of the phosphopeptides of varying degrees of

<sup>8</sup> Lipmann, F. A., *Naturwissenschaften*, **21**, 236 (1933).

complexity—a method which will be applied in this laboratory for a further and more detailed study of the so called phosphopeptones.

#### EXPERIMENTAL

*Digestion of Casein by Trypsin*—A solution of 50 gm. of casein in 200 cc. of water and 40 cc. of *N* sodium hydroxide was well shaken and the volume made up to 500 cc. with distilled water. Sodium carbonate was added to pH 8.5 and the solution allowed to stand with 1.5 gm. of trypsin (Fairchild) for 2 days at 37°. The solution was then filtered, the filtrate made alkaline to phenolphthalein with barium hydroxide solution, and the precipitated barium phosphate centrifuged out. The clear solution was faintly acidified with 10 per cent neutral lead acetate until no further precipitation of lead salts occurred. The lead salts were centrifuged and the mother liquors discarded. The solid was stirred with 10 per cent sodium carbonate until the solution was faintly alkaline to phenolphthalein and the insoluble lead carbonate was centrifuged out and discarded. The clear solution containing the sodium salt of the phosphopeptone was then treated with 3 volumes of alcohol and the precipitate so obtained allowed to settle. The sodium salt of the phosphopeptone was thus obtained as a syrup which could be solidified by prolonged stirring with acetone. It was redissolved and reprecipitated for purification. No attempt was made to fractionate the product or to purify it further. Obtained in this way the material from three different experiments had the following N:P ratios, (a) 3.83, (b) 4.11, (c) 4.30.

*Rate of Hydrolysis of Phosphopeptone by 2 N Hydrochloric Acid*—Sealed tubes, each containing 200 mg. of phosphopeptone dissolved in 2 cc. of 2 *N* hydrochloric acid were heated for varying periods at 100° and the increase in amino nitrogen and inorganic phosphorus followed. For this the solutions were diluted to 25 cc. of which 2 cc. were used for the amino nitrogen estimation (Van Slyke) and 15 cc. for the determination of inorganic phosphorus. The latter was precipitated by magnesia mixture, filtered, redissolved in dilute hydrochloric acid, and estimated colorimetrically by the method of King.<sup>9</sup> The rate of formation of each is given in Table I. It was not found possible to hydrolyze further than

<sup>9</sup> King, E. J., *Biochem. J.*, **26**, 292 (1932).



80 per cent amino nitrogen by this method. Subsequently, the purest fractions of the phosphoamino acid isolated through the barium salt were hydrolyzed as shown later but with no increase in the amino nitrogen.

*Hydrolysis of Phosphopeptone by 2 N Hydrochloric Acid*—Two 500 cc. Pyrex flasks each containing 20 gm. of phosphopeptone and 200 cc. of 2 N hydrochloric acid were sealed and heated in the steam bath for 10 hours. The acid solutions were combined and

TABLE I  
*Rate of Increase in Amino Nitrogen and Inorganic Phosphorus at 100°*

Time	Amino N Total N	Inorganic P Total P
<i>hrs</i>	<i>per cent</i>	<i>per cent</i>
Initial	13 30	
2	53 03	7 3
4	67 34	11 9
6	78 32	
8	78 32	
10	79 62	22 4

TABLE II  
*Fractionation of Barium Salt by Alcohol*

Alcohol	N	P	NH <sub>2</sub> -N	N/P	Weight
<i>cc</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		<i>gm.</i>
200	3 542	4 89	2 572	1 6	2 0
400	4 256	5 30	2 803	1 7	2 8
800	4 676	4 27	2 890	2 4	2 6
1600	4 704	2 95	2 688	3 5	0 6
Total weight					8 0

neutralized to phenolphthalein by barium hydroxide. The white precipitate so obtained was centrifuged, dissolved in dilute acetic acid, and the solution neutralized to litmus by dilute ammonia. The precipitate which formed was discarded and the clear solution added to the mother liquor above. The whole was neutralized to phenolphthalein by barium hydroxide and the solution treated with an equal volume of alcohol. The precipitated barium salt was centrifuged and, since it still contained a little chloride, was

redissolved in 1600 cc. of water and reprecipitated with alcohol in portions, as shown in Table II.

*Further Hydrolysis of Barium Salt by Hydrochloric Acid*—The first barium salt isolated as described above had a total nitrogen to amino nitrogen ratio of 1.37 and it was thought that further hydrolysis would reduce this to 1.0. This proved not to be the case. (a) 200 mg. portions were dissolved in 2 cc. of 2 N hydrochloric acid and allowed to stand at room temperature in sealed tubes for

TABLE III  
*Variation of Total and Amino Nitrogen on Hydrolysis of Barium Salt*

Time	Total N	Amino N	Total N Amino N
hrs.	mg.	mg.	
3	7 945	5 922	1 34
6	8 260	6 003	1 37
9	7 770	5 954	1 29
24	7 875	6 204	1.27
32	7 700	5 737	1 34

TABLE IV  
*Total and Amino Nitrogen Values during Hydrolysis of Barium Salt*

Time	Total N	Amino N	Total N Amino N
hrs	mg	mg	
Initial	9 100	4 959	1 83
3	8 155	6 130	1 33
5	8 960	6 619	1 35
10	8 750	7 520	1 17

varying periods. Total and amino nitrogen values were then determined and no further hydrolysis could be detected. This is shown in Table III. Another 200 mg. were heated in the steam bath under similar conditions. After 4 hours it had a total nitrogen to amino nitrogen ratio of 1.24. (b) In a later experiment 10 gm. of barium salt with a N:P ratio of 2.1 were dissolved in 100 cc. of 2 N hydrochloric acid and heated under a reflux. Samples were taken at intervals and the total and amino nitrogen values estimated. The results are shown in Table IV. Unfortunately, after 10 hours of hydrolysis the majority of the phosphorus had

been converted to phosphoric acid and it was only possible to isolate from the above experiment 1.5 gm. of barium salt and this had a N:P ratio of 1.36.

*Purification Through the Brucine Salt. Isolation of Brucine Salt*—By a careful and tedious fractionation of the barium salt it was possible to obtain salts with N:P ratios of 1.25 to 1.30, but fractionation beyond this point was not successful. Other means of purification were therefore sought and in the brucine salt a completely successful one was found. The barium salt was dissolved in a little dilute acetic acid and dilute sulfuric acid was added drop by drop until precipitation was almost complete. The barium sulfate was centrifuged and the clear solution treated with neutral lead acetate until no further precipitation occurred. The lead salt was centrifuged and well washed with water. It was then suspended in water and decomposed with hydrogen sulfide. The sulfide was filtered and washed and the filtrate aerated to remove hydrogen sulfide. When free, it was evaporated under reduced pressure to a low bulk and treated with brucine dissolved in a small volume of alcohol. The aqueous alcoholic solution was warmed for a few minutes and then allowed to cool. The salt did not separate and the alcohol was removed under reduced pressure and the salt crystallized from aqueous acetone. It was recrystallized from the same solvent and was obtained as colorless needles of melting point 171–172°. Dried *in vacuo* at 80° for analysis, it gave figures in agreement with brucine glutamylserinephosphate.

6.535 mg. substance: 0.461 cc. N (27°, 748 mm.)

5.175 “ “ : 13.480 mg. ammonium phosphomolybdate

$C_{31}H_{39}O_{13}N_4P$ . Calculated. N 7.93, P 4.37

Found. “ 7.90, “ 3.78

*Barium Salt of Phosphodipeptide*—2 gm. of the brucine salt described above were dissolved in a small volume of water and the solution neutralized to phenolphthalein by barium hydroxide. The precipitated brucine was filtered and the filtrate extracted with chloroform. The aqueous solution was again made alkaline to phenolphthalein with barium hydroxide, evaporated to 25 cc., the barium salt precipitated by addition of alcohol, and centrifuged. The solid was shaken with 50 cc. of water, the solution filtered, and half its volume of alcohol added. The barium salt

was centrifuged, washed with alcohol and ether successively, and dried at 80° *in vacuo* for analysis. It gave figures agreeing with tribarium glutamylserinephosphate.

7.030 mg. substance:	0.332 cc. N (22.5°, 755 mm.)
4.665 " "	: 19.380 mg. ammonium phosphomolybdate
52.600 " "	: 37.400 " BaSO <sub>4</sub>
31.800 " "	: 1.63 cc. N (22.5°, 755 mm.)
(C <sub>8</sub> H <sub>10</sub> O <sub>5</sub> N <sub>2</sub> PBa) <sub>2</sub> Ba. Calculated.	N 5.43, P 6.02, Ba 40.00, NH <sub>2</sub> -N 2.71
Found.	" 5.42, " 6.16, " 41.80, " 2.87

*Hydrolysis of Brucine Salt*—An aqueous solution of 12 gm. of the brucine salt was made alkaline with barium hydroxide and the precipitated brucine filtered off. The filtrate was extracted with chloroform and evaporated under reduced pressure to 70 cc. Concentrated hydrochloric acid was then added in sufficient quantity to make a 20 per cent acid solution and it was heated under a reflux for 6 hours, by which time hydrolysis was complete. The barium in the solution was removed quantitatively by sulfuric acid and the solution evaporated many times at reduced pressure to remove the hydrochloric acid. The residue was dissolved in 150 cc. of water and an excess of calcium hydroxide added in the form of a cream. The excess was removed together with calcium phosphate and the clear solution treated with 4 volumes of alcohol. The sticky precipitate of calcium salts was centrifuged and dissolved in 100 cc. of water. Calcium was removed quantitatively by oxalic acid and the solution evaporated under reduced pressure until crystallization commenced. The solid was filtered off, washed with alcohol, and analyzed after drying in a vacuum over phosphorus pentoxide. It proved to be glutamic acid.

4.526 mg. substance:	6.340 mg. CO <sub>2</sub> and 2.800 mg. H <sub>2</sub> O
50.000 " "	: 2.98 cc. 0.1 N HCl
C <sub>5</sub> H <sub>9</sub> O <sub>4</sub> N. Calculated.	C 40.81, H 6.20, N 9.52
Found.	" 40.80, " 7.37, " 8.91

A portion of this acid was converted to the hydrochloride which was crystallized from concentrated hydrochloric acid. It was analyzed after drying in a vacuum over phosphorus pentoxide and soda-lime. It had a melting point of 213° (m.p. of glutamic acid hydrochloride, 214°). The substance had the following composition.

5.020 mg. substance: 5.980 mg. CO<sub>2</sub> and 2.560 mg. H<sub>2</sub>O

50.000 " " : 2.69 cc. 0.1 N HCl

6.405 " " : 5.082 mg. AgCl

C<sub>8</sub>H<sub>10</sub>O<sub>4</sub>NCl. Calculated. C 32.69, H 5.45, N 7.62, Cl 19.34

Found. " 32.47, " 5.70, " 7.53, " 19.62

The alcoholic mother liquors from the calcium salt were evaporated and the solid residue taken up in a little water. Calcium was removed quantitatively by oxalic acid and the solution evaporated. Difficulty was experienced in purifying the crude serine so obtained and it was therefore isolated as the  $\beta$ -naphthalenesulfonyl derivative by the method of Fischer and Bergell.<sup>10</sup> After recrystallizing from water it had a melting point of 209°. It had the following composition.

100.0 mg. substance: 3.28 cc. 0.1 N HCl

C<sub>18</sub>H<sub>13</sub>O<sub>5</sub>NS. Calculated, N 4.74; found, N 4.59

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<sup>10</sup> Fischer, E., and Bergell, P., *Ber. chem. Ges.*, **35**, 3779 (1902).

# THE GROWTH-PROMOTING PROPERTIES OF HOMOCYSTINE WHEN ADDED TO A CYSTINE-DEFICIENT DIET AND THE PROOF OF STRUCTURE OF HOMOCYSTINE

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In a recent investigation by Butz and du Vigneaud (1) upon the isolation of the next higher symmetrical homologue of cystine from the decomposition of methionine by  $H_2SO_4$ , it was pointed out that the study of the utilization of this homologue for growth purposes would be particularly interesting in view of the findings of Jackson and Block (2) with respect to methionine. These investigators have shown that the feeding of methionine to animals on a cystine-deficient diet produced a marked increase in their rate of growth. One might expect that this homologue of cystine, homocystine, should also support growth on a cystine-deficient diet if demethylation takes place during the metabolism of methionine.

The evidence offered in the paper mentioned above for the structure of homocystine, bis-( $\gamma$ -amino- $\gamma$ -carboxypropyl) disulfide, was based on the behavior and analysis of the compound itself and a number of its derivatives such as dibenzoylhomocystine, benzylhomocysteine, and homocysteic acid. It was well recognized, however, that this could not definitely prove the structure; only actual synthesis of the compound, or its conversion to another compound whose structure had already been proved by synthesis, could suffice. This final proof of structure has now been established by conversion of homocystine to methionine by reduction of the homocystine in liquid ammonia with metallic sodium and methylation of the homocysteine so formed by the addition of methyl-iodide to the liquid ammonia solution of the compound according

to the method worked out by du Vigneaud, Audrieth, and Loring (3) for the formation of benzylcystine.

#### EXPERIMENTAL

##### *Conversion of Homocystine to Methionine*

0.5 gm. of homocystine prepared according to the directions of Butz and du Vigneaud (1) was dissolved in approximately 30 cc. of liquid ammonia. Enough sodium was added in small pieces just to give a blue color indicating a slight excess of sodium. To this solution was added with stirring 0.6 gm. of methyl iodide. The ammonia was then allowed to evaporate spontaneously. The white residue was then dissolved in 10 cc. of water, acidified to Congo red with 30 per cent HBr, and the solution filtered. To the filtrate 1 cc. of pyridine and 3 volumes of boiling ethyl alcohol were added and the mixture cooled in an ice bath. The precipitate that formed was filtered and washed with a mixture of 3 parts of alcohol and 1 part of water. The melting point of this product was 268–271°. The precipitate was then dissolved in 6 cc. of water with heating and 3 volumes of hot alcohol added. As the solution cooled glistening plate-like crystals separated out identical in appearance and behavior with an authentic sample of synthetic *dl*-methionine recrystallized in the same way. The yield of the recrystallized product was 0.214 gm., the melting point of which was 275–276° (corrected). Further recrystallization did not raise the melting point. The melting point of the authentic specimen was 275–276° (corrected). A mixture of the two melted at 275–276° (corrected).

##### *Analysis*

2.663 mg. substance: 0.218 cc. N at 25° and 761 mm.

3.103 " " : 0.253 " " " 25° " 761 "

$C_5H_{11}O_2NS$ . Calculated, N 9.39; found, N 9.41, 9.35

##### *Growth Experiments*

In our first experiments we attempted to use the diet (Diet II) described in the studies on the utilization of *d*- and *l*-cystine (4). This diet had the following composition: casein 8.0, dextrin 31.0, sucrose 15.0, lard 19.0, cod liver oil 5.0, salt mixture (Osborne and Mendel (5)) 4.0, agar 2.0, and milk vitamin concentrate 16.0. Although in those experiments the animals on the

basal diet grew only slightly if at all, we found that in the present investigation our rats on the basal diet grew at too great a rate with the result that the addition of cystine to the diet did not produce a sharp enough difference between the control and the ex-

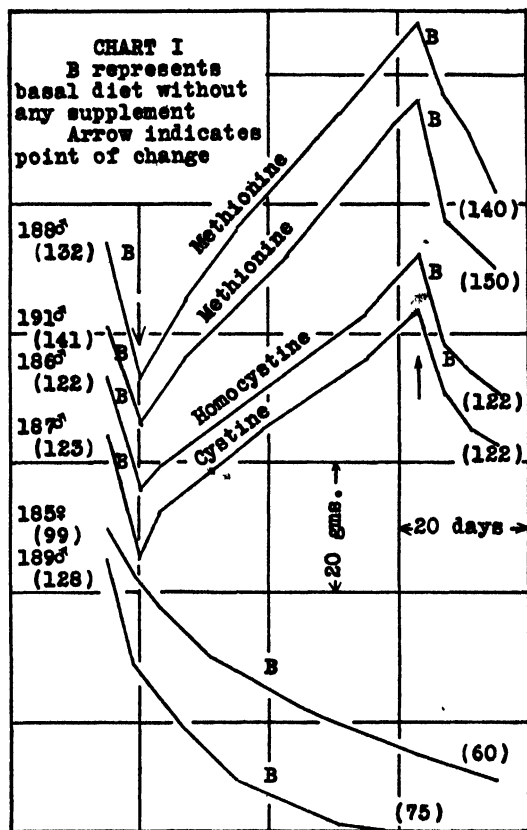


CHART I. The numbers in parentheses denote the initial and final weights of the rats. The No. and sex of each rat are shown on the extreme left.

perimental rats. It was therefore necessary to modify the diet in order to produce a clean cut difference in the growth rate of the animals on the basal diet and of those with the cystine supplement. After numerous trials a diet was made up as follows: casein 5.0, dextrin 38.0, sucrose 15.0, lard 19.0, cod liver oil 5.0, salt mixture



(Osborne and Mendel (5)) 4.0, agar 2.0, and milk vitamin concentrate 12.0.<sup>1</sup> All the animals placed on this diet without exception not only failed to grow but lost weight and the addition of cyst-

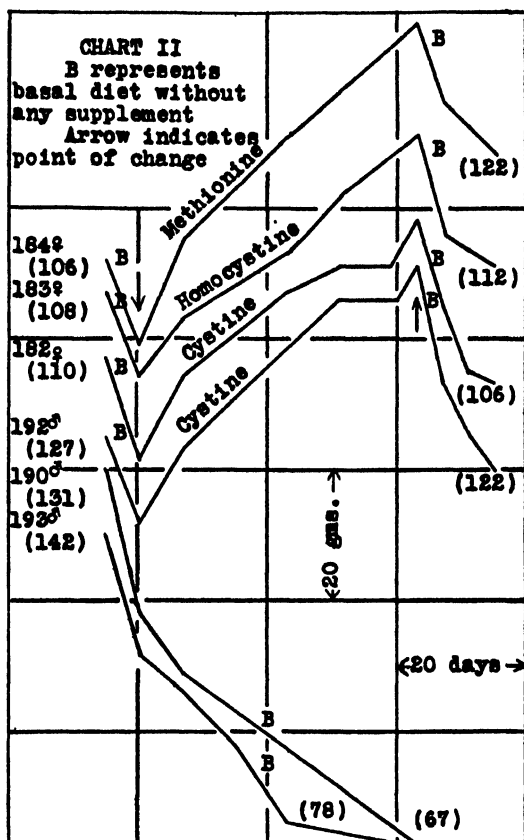


CHART II. The numbers in parentheses denote the initial and final weights of the rats. The No. and sex of each rat are shown on the extreme left.

ine to the diet, either incorporated with the basal diet or fed as separate pills, caused an immediate resumption of growth. This

<sup>1</sup> The authors wish to thank Dr. Supplee of the Research Laboratories of The Dry Milk Company, Inc., for a liberal supply of the milk vitamin concentrate which was used in this investigation.

diet was therefore used for our comparison of inactive homocystine with *l*-cystine and with *dl*-methionine. In preliminary experiments it was found that the rats would not consistently eat pills containing the homocystine. It was necessary to incorporate the homocystine in the diet. The same was therefore done with the cystine and the methionine. For the feeding of cystine, 0.3 gm. of cystine was added to 100 gm. of diet while twice the

TABLE I  
*Food Consumption and Body Weight Changes*

Rat No. and sex	Days	Supplement* to basal diet per day	Average daily food consumption	Rat No and sex	Days	Supplement* to basal diet per day	Average daily food consumption
		mg	gm			mg	gm
185 ♀	1-64		5 0	183 ♀	1- 5		6 6
189 ♂	1-64		5 9		5-48	58 <i>dl</i> -homocystine	8 6
190 ♂	1-64		5 9		48-64		7 1
193 ♂	1-64		6 2	186 ♂	1- 5		7 8
182 ♀	1- 5		7 0		5-48	66 <i>dl</i> -homocystine	9 7
	5-48	27 <i>l</i> -cystine	8 8		48-64		7 5
	48-64		7 9	184 ♀	1- 5		7 0
187 ♂	1- 5		7 0		5-48	65 <i>dl</i> -methionine	8 7
	5-48	27 <i>l</i> -cystine	9 1		48-64		6 9
	48-64		6 5	188 ♂	1- 5		8 2
192 ♂	1- 5		7 6		5-48	77 <i>dl</i> -methionine	10 3
	5-48	27 <i>l</i> -cystine	9 0		48-64		6 7
	48-64		7 6	191 ♂	1- 5		10 0
					5-48	80 <i>dl</i> -methionine	10 7
					48-64		6 9

equivalent amount of homocystine, 0.675 gm., was used. This was done because of the fact that the homocystine was optically inactive and of course the behavior of the individual isomers was not known. In the case of methionine 0.750 gm. was employed per 100 gm. of basal diet, in order to give a direct comparison between the homocystine and methionine. After these experiments were initiated Jackson and Block (6) demonstrated that both *d*- and *l*-methionine could support growth, so that we were

actually feeding utilizable methionine at a higher level than the cystine. Substitution of an amount of nitrogen in the form of casein equivalent to the homocystine did not prevent the fall in weight on the basal diet.

Three litters in all were used in the growth experiments with homocystine, two litters of twelve animals each and one of four being used. Since the results consistently demonstrated that homocystine could support growth in lieu of cystine or methionine, we shall report in detail the results with only one litter for the sake of brevity.

This litter of twelve rats was placed on the basal diet for 5 days. At the end of this period three of the rats were given the basal diet plus cystine, three more basal plus methionine, and two rats were given the basal diet plus homocystine, the supplements being incorporated with the basal diet in the amounts mentioned above. Four of the twelve rats were kept as controls. The results are shown in Charts I and II and the food consumption in Table I.

#### DISCUSSION

Our results show unmistakable stimulation of growth when homocystine is added to the cystine-deficient diet and furthermore confirm the findings of Jackson and Block (2) on methionine. There is not as yet, however, sufficient experimental evidence fully to evaluate this growth-stimulating power of either methionine or homocystine. The various possibilities with respect to methionine have already been amply discussed by Jackson and Block (2) and as they indicate, further work is necessary to demonstrate whether or not methionine can actually be converted to cystine in the body. These same considerations also apply to homocystine. The present investigation, however, was not concerned directly with this phase of the problem but was designed rather to see if homocystine could support growth on a cystine-deficient diet and to bring evidence to bear on the possible relationship between methionine and homocystine in the body.

The results we feel are in harmony with the idea that demethylation may occur in the intermediary metabolism of methionine with the formation of homocysteine. It must of course be admitted that one may be dealing here with a non-specific action of homocystine in simply supplying sulfur to the organism in a form

that can be utilized rather than with any direct relationship between it and the other compounds mentioned. This seems, however, improbable, particularly in view of the fact that other disulfide acids have been tried for promotion of growth of animals on cystine-deficient diets with completely negative results. Dithioglycollic acid,  $\beta$ -dithiopropionic acid, and  $\alpha$ -hydroxy- $\beta$ -dithiopropionic acid were found to be unable to support growth by Westerman and Rose (7) in spite of the fact that the body could oxidize these compounds (8).

The observation of Jackson and Block (6) that both *d*- and *l*-methionine can support growth and our own observation (4) that *d*-cystine cannot replace the levo isomer make all the more desirable a study of the optical isomers of homocystine from this standpoint. The homocystine used in our experiments was optically inactive and was fed at twice the equivalent level of the cystine. As will be noted the rate of growth obtained with this higher level of inactive homocystine was strikingly the same as that produced by the *l*-cystine. On the other hand the *dl*-methionine which was also fed at twice the level of the sulfur of *l*-cystine gave rise consistently to a greater rate of growth than the cystine. The results with *dl*-methionine are now easily explainable on the basis of the results of Jackson and Block since both isomers of methionine should be effective and therefore should have given a greater stimulation of growth if the amount of cystine used was less than the amount necessary to produce a maximum rate of growth. The fact though that inactive homocystine did not produce a greater rate of growth than the *l*-cystine which was fed in an amount one-half as great as the homocystine equivalent might be an indication that both isomers of homocystine unlike methionine may not be equally available for growth. The completeness of oxidation of this inactive homocystine in contrast to the incomplete oxidation of *d*-cystine, which we have recently found (9), raises some doubt as to such a conclusion. We would prefer, however, to leave this matter to be settled by studies on the pure isomers which we hope to carry out.

#### SUMMARY

The growth-promoting properties of homocystine when added to a cystine-deficient diet have been demonstrated and the possible

relationship between homocystine and methionine in intermediary metabolism has been discussed.

The structure of homocystine as previously given has been substantiated by conversion of homocystine to methionine by reduction and subsequent methylation.

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## THE OXIDATION OF CYSTINE IN NON-AQUEOUS MEDIA

### II. STUDIES ON THE HYDRATION OF ACETONITRILE AND ACETIC ANHYDRIDE BY A NON-AQUEOUS TITRATION METHOD\*

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A method of preparing a non-aqueous solution of cystine cation by using aqueous 70 per cent perchloric acid in acetonitrile has been previously described (1). Water was removed by acetic anhydride; but under certain conditions, it seemed to react with acetonitrile. The present paper deals with a titrimetric investigation of the rates of these two reactions.

#### *I. Method of Titration*

Folin (2-4) showed that even very weak acids may be successfully titrated by an alcoholate in non-aqueous media. We found that with methyl alcoholic sodium methylate as a base a sharp differentiation may be obtained between strong and weak acids by the use of thymolsulfonephthalein (thymol blue). This indicator in a suitable non-aqueous medium does not change from red to yellow until the strong acids present are completely neutralized while weak acids are completely neutralized between this end-point and the second color change from yellow to blue. The color given to non-basic or very slightly basic solvents,<sup>1</sup> such as chloroform or acetonitrile, depends on the form of the added indicator; red with the acid and yellow with the salt. In more basic solvents such as alcohols and  $H_2O$  the yellow ion is formed regardless of which form is added. Methyl alcohol requires a correction for its in-

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† Daniel Wentz Fellow of the Lankenau Hospital Research Institute.

<sup>1</sup> The concepts of acidity and basicity are used in accordance with the formulation of Brönsted (5).

herent acidity. Standardization values for the methylate solution are given in Table I. The value obtained from acetic anhydride is based on the observation that on non-aqueous titration the anhydride reacts according to  $(\text{CH}_3\text{CO})_2\text{O} + \text{CH}_3\text{O}^- \rightarrow \text{CH}_3\text{COO}^- + \text{CH}_3\text{COOCH}_3$  as shown by the following titrations. 2.195 mm  $(\text{CH}_3\text{CO})_2\text{O} + 0.158$  mm  $\text{CH}_3\text{COOH}^2$  in 15 cc. of  $\text{CHCl}_3$  consumed 2.355 mm of 0.301 N  $\text{NaOCH}_3$ ; calculated, 2.353 mm. The presence of several equivalents of  $\text{H}_2\text{O}$  does not affect this reaction. 2.100 mm  $(\text{CH}_3\text{CO})_2\text{O} + 0.153$  mm  $\text{CH}_3\text{COOH} + 5.5$  mm  $\text{H}_2\text{O}$  in 10 cc. of  $\text{CHCl}_3$  were equal to 2.250 mm; calculated, 2.253 mm. In fact when 70 per cent methyl alcohol is present, acetic anhydride consumes only 1 equivalent of base even on titration with aqueous

TABLE I  
*Standardization of  $\text{NaOCH}_3$*

Standard	Indicator	Medium at end-point			$\text{NaOCH}_3$
		$\text{H}_2\text{O}$	$\text{CH}_3\text{OH}$	$\text{CHCl}_3$	
		cc	cc.	cc.	N
HCl	Phenolphthalein	20	10		0 1191 0 1195
"	"	40	10		0 1192
$\text{C}_6\text{H}_5\text{COOH}$	Thymol blue		17	20	0.1192
$(\text{CH}_3\text{CO})_2\text{O}$	" "		14	25	0 1189

alkali. 1.143 mm  $(\text{CH}_3\text{CO})_2\text{O} + 0.083$  mm  $\text{CH}_3\text{COOH}$  in 50 cc. of  $\text{CH}_3\text{OH}$  used 1.223 mm of 0.05 N  $\text{NaOH}$ ; calculated, 1.226 mm. Similarly: found, 1.010 mm; calculated, 1.015 mm.

If we assume acceleration by basic catalysis (5) these observations can be explained by the work of Caudri (6) who found that the alcoholysis constant of acetic anhydride is greater than the hydrolysis constant, both in aqueous and in methyl alcoholic solutions.

The titration of perchloric acid is illustrated in Table II. Column 2 gives the weight of  $\text{HClO}_4$  present according to aqueous standardization. Samples 1 to 4 refer to Kahlbaum Analytical (69.73 per cent)  $\text{HClO}_4$ , Samples 5 and 6 to Merck's Blue Label (67.47 per cent)  $\text{HClO}_4$ . Column 3 denotes the amount titrated at the first color change (red to yellow). Column 4 gives the per-

<sup>2</sup> The  $(\text{CH}_3\text{CO})_2\text{O}$  contained 4.1 per cent  $\text{CH}_3\text{COOH}$ .

centage deviation of the found values from the calculated ones. Column 5 gives the amount of  $\text{HClO}_4$  estimated from the second color change (yellow to blue). Since dimethyl yellow has but one color change (red to yellow), thymol blue was added after the first end-point was reached (Column 6). Columns 7 to 9 give the volume and composition of the medium at the end of the titration.

Except possibly in pure  $\text{CH}_3\text{OH}$  the results are about 1.5 per cent too low. The presence of any related weak acid could not be detected and seems unlikely since two different commercial products gave quite similar results. The sensitivity of the indicator (thymol blue) to  $\text{HClO}_4 + 2\frac{1}{2}\text{H}_2\text{O}^3$ , determined by dilution

TABLE II  
*Titration of  $\text{HClO}_4 + 2\frac{1}{2}\text{H}_2\text{O}$*

Sample No. (1)	$\text{HClO}_4$ calculated (2)	Red to yellow $\text{HClO}_4$ found (3)	Deviation (4)	Yellow to blue $\text{HClO}_4$ found (5)	Indicator (6)	Medium at end-point		
						$\text{CH}_3\text{OH}$ (7)	$\text{CHCl}_3$ (8)	$\text{CH}_3\text{CN}$ (9)
	gm.	gm.	per cent	gm.		cc.	cc.	cc.
1	0 1780	0 1755	1 4	0 1779	Thymol blue	14	15	15
2	0 1607	0 1584	1 4	0 1607	Dimethyl yellow	12	15	
3	0 1713	0 1683	1 7	0 1713	" "	14		
4	0 2250	0 2239	0 5	0 2257	Thymol blue	25		
5	0 1056	0 1043	1 2	0 1057	" "	9	15	
6	0 0872	0 0861	1 3	0 0869	" "	7	18	

with the following solvents, and expressed by the number of mols per liter required to produce a distinct red color, is as follows:  $4.7 \times 10^{-6}$  in  $\text{CHCl}_3$ ,  $3 \times 10^{-5}$  in  $\text{CH}_3\text{OH}$ . The indicator concentration (Na-salt) was  $6 \times 10^{-6}$  mols per liter. The fact that the use of 70 per cent perchloric acid entails a water concentration of 0.1 to 0.2 M in the titrations does not account for the low results since addition of  $\text{H}_2\text{O}$  up to 0.35 M does not discharge the red indicator color in a  $4 \times 10^{-5}$  N solution of  $\text{HClO}_4$  in a 1:1 mixture of  $\text{CHCl}_3$  and  $\text{CH}_3\text{OH}$ .

A definite explanation of the deviations might require a more fundamental study of salt effects in these media. This is beyond the scope of our present problem and we have been content with

<sup>3</sup> Perchloric acid is marketed as approximately 70 per cent  $\text{HClO}_4$  which corresponds roughly to  $\text{HClO}_4 + 2\frac{1}{2}\text{H}_2\text{O}$ .



the accuracy obtained, applying empirical corrections when necessary.

Estimation of perchloric acid and acetic acid, present together in a molar ratio of 1:5 in acetonitrile solutions, is just as accurate as when titrated separately, if the solution is diluted with  $\text{CHCl}_3$  or  $\text{CH}_3\text{OH}$ . This point is illustrated by Table III, to be discussed in Part II.

### II. Reaction between Water and Acetonitrile

In the preceding paper (1) the following reactions were postulated.



and

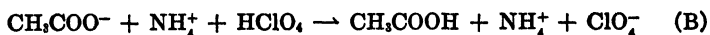


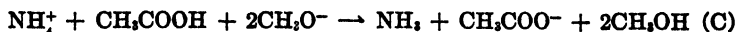
TABLE III

*Reaction between  $\text{CH}_3\text{CN}$ ,  $\text{H}_2\text{O}$ , and  $\text{HClO}_4$*

$\text{HClO}_4$ , 0.1964 N;  $\text{H}_2\text{O}$ , 0.5193 M.

Time	$\text{HClO}_4$	Decrease in $\text{HClO}_4$	$\text{NH}_4^+ + \text{CH}_3\text{COOH}$	
			Calculated	Found
hrs	N	N	N	N
24	0 1901	0 0063	0 0126	0 0125
96	0 1550	0 0414	0 0828	0 0826
216	0 0751	0 1213	0 2426	Crystals pre- cipitated

Titration of a weighed sample of  $\text{CH}_3\text{COONH}_4$  in a  $\text{CHCl}_3$ - $\text{CH}_3\text{OH}$  mixture with  $\text{HClO}_4$  in  $\text{CH}_3\text{CN}$  consumed 1 equivalent of  $\text{HClO}_4$  at the change of thymol blue from yellow to red demonstrating the quantitative nature of Reaction B in the non-aqueous medium. Titration of the resulting solution to the blue indicator color corresponded to the reaction



The evolution of  $\text{NH}_3$  was observed.

To demonstrate the validity of Reaction A a solution of 70 per cent  $\text{HClO}_4$  in acetonitrile was kept at 50–60°. 4 cc. portions were

withdrawn at intervals, diluted with 20 cc. of  $\text{CHCl}_3$ , and titrated with  $\text{NaOCH}_3$ . The results are given in Table III. The reaction corresponds to Reaction A + Reaction B.



Theoretically the decrease in  $\text{HClO}_4$  obtained by the first end-point should be equal to one-half the sum of  $\text{NH}_4^+$  and  $\text{CH}_3\text{COOH}$  obtained by the second end-point.

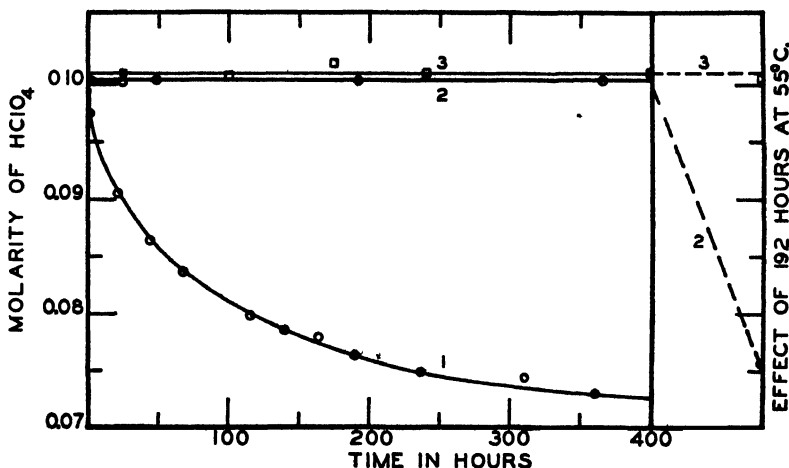


FIG. 1. The reaction between water and acetonitrile. The composition of the solutions in mol per liter is as follows:

Curve No	$\text{HClO}_4$	Cystine	$\text{H}_2\text{O}$	HCN
1	0.1004		0.265	0.0331
2	0.1004		0.265	
3	0.1012	0.0506	0.267	

The crystals were identified as ammonium perchlorate by precipitation of  $\text{KClO}_4$  in alcoholic solution, by  $\text{NH}_3$  evolved by the action of aqueous  $\text{NaOH}$ , and by titration with  $\text{NaOCH}_3$ . 0.0902 gm. of crystals, calculated as  $\text{NH}_4\text{ClO}_4$ , 0.768 mm; found, 0.768 mm.

Fig. 1 shows the behavior of various solutions kept at  $32.50^\circ$ .

*Solution 1*—The acetonitrile used contained HCN which is also neutralized by methylate. The rate of decrease of  $\text{HClO}_4$  is

decidedly different from that in the HCN-free solutions. Although this points to the hydrolysis of HCN rather than of  $\text{CH}_3\text{CN}$ , formic acid could not be detected. The fact that the blank titration of HCN must be deducted throughout the experiment to make the weak acid values agree with the decrease of  $\text{HClO}_4$  also speaks against the hydrolysis of HCN. However, it is evident that HCN or other unidentified impurities affect the hydrolysis of  $\text{CH}_3\text{CN}$  in some manner.

*Solution 2*—The composition is the same except that it contains no HCN. In this solution the weak acid values were practically constant at 2 per cent of the total and equal to the apparent loss in  $\text{HClO}_4$ . This may be accounted for by the salt effect deficit (see Part I) in the titration of the initial  $\text{HClO}_4$ . In Solutions 1 and 2, 5 cc. samples were titrated after dilution with 15 cc. of  $\text{CHCl}_3$ .

*Solution 3*—On titrating cystine perchlorate the first color change corresponds to the conversion of  $\text{R}(\text{NH}_3^+)_2(\text{COOH})_2$  to  $\text{R}(\text{NH}_3^+)_2(\text{COO}^-)_2$  and the second change to the conversion into  $\text{R}(\text{NH}_2)_2(\text{COO}^-)_2$ . 4 cc. portions were diluted with 10 cc. of  $\text{CHCl}_3$  near the first end-point, and after passing it, 5 cc. of  $\text{CH}_3\text{OH}$  were added. In this manner the difficulties caused by precipitation were minimized. The points on Fig. 1 represent one-half the total titration.

To bring out any difference in stability, Solutions 2 and 3 were heated at  $50\text{--}60^\circ$  for 192 hours with the results also shown in Fig. 1. The cystine cation, although a relatively strong acid, evidently does not catalyze the hydrolysis of  $\text{CH}_3\text{CN}$ . In fact, a 0.5 *N* cystine perchlorate solution stood for 6 months without precipitation of cystine. If  $\text{CH}_3\text{COONH}_4$  were formed it should precipitate cystine,  $\text{R}(\text{NH}_3^+)_2(\text{COOH})_2 + 2\text{CH}_3\text{COO}^- \rightarrow 2\text{CH}_3\text{COOH} + \text{R}(\text{NH}_3^+)_2(\text{COO}^-)_2$ . This was experimentally verified by adding  $\text{CH}_3\text{COONH}_4$  to a cystine perchlorate-acetonitrile solution, whereupon cystine was precipitated.

$\text{H}_2\text{O} + \text{CH}_3\text{CN}$ —Over a period of 20 days solutions of water (0.264 *M*) in pure acetonitrile showed no tendency to interact regardless of whether HCN was present or not.

### III. Reaction between Water and Acetic Anhydride

The preceding paper (1) indicated that under the experimental conditions the reaction between water and acetic anhydride was

quite rapid. Definite proof was obtained by following titrimetrically the change in acidity resulting from the conversion of 1 mol of  $(\text{CH}_3\text{CO})_2\text{O}$ , which on titration requires 1 mol of methylate (*cf.* Part I) to 2 mols of  $\text{CH}_3\text{COOH}$  (2 mols of methylate).

Fig. 2 shows the rate of hydration of acetic anhydride in two solutions of cystine perchlorate in acetonitrile, and for comparison

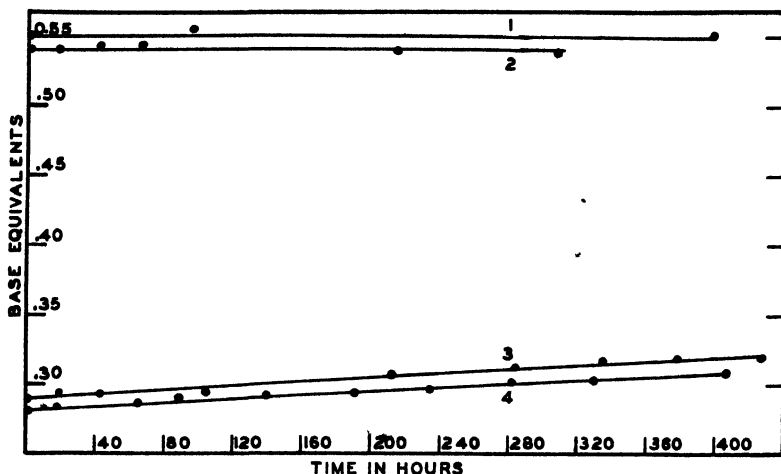


FIG. 2. The reaction between water and acetic anhydride. The composition of the solutions in mol per liter is as follows.

Curve No	Cystine perchlorate	$\text{H}_2\text{O}$	$(\text{CH}_3\text{CO})_2\text{O}$	Calculated base equivalents		HCN
				Initial*	For complete hydration	
1	0.0501	0.2645	0.2645	0.284	0.548	
2	0.0493	0.260	0.260	0.279	0.539	0.033
3		0.264	0.264	0.284	0.548	0.033
4		0.516	0.258	0.277	0.535	

\* The  $(\text{CH}_3\text{CO})_2\text{O}$  contained 4.1 per cent  $\text{CH}_3\text{COOH}$ .

the rate in two corresponding solutions which contain no cystine perchlorate. 2 cc. portions diluted with 10 cc. of  $\text{CHCl}_3$  were titrated. The ordinate, base equivalents, represents, in mols per liter, the combined acidity of  $(\text{CH}_3\text{CO})_2\text{O}$  and  $\text{CH}_3\text{COOH}$ , calculated from the total titrations by deducting for cystine (and HCN, if present). The HCN present in one pair of solutions did not

affect the rate of hydration. The cystine perchlorate solutions were completely dehydrated 1 hour after preparation when the first sample was withdrawn, while the blank solutions were only 11.5 per cent dehydrated after 400 hours.

The authors wish to take this opportunity to acknowledge the helpful advice and friendly criticism of Professor M. Kilpatrick, Jr.<sup>4</sup>

#### SUMMARY

The two color changes of thymol blue were found to distinguish between strong and weak acids in non-aqueous titrations with sodium methyrate as the base and chloroform as diluent. Acetic anhydride can be titrated in the same way, reacting mol for mol with sodium methyrate.

The titration was used to study the following conditions met in the preparation of solutions of cystine perchlorate in acetonitrile: (1) the hydrolysis of acetonitrile; (2) the rate of dehydration by means of acetic anhydride.

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<sup>4</sup> A report of determinations of acid strengths in  $\text{CH}_3\text{CN}$ , carried out at the Marine Experimental Station of the Lankenau Hospital Research Institute, will be published by Mary L. Kilpatrick and Martin Kilpatrick, Jr.

## THE METABOLISM OF SULFUR

### XX. THE RATE OF ABSORPTION OF *dl*-METHIONINE FROM THE GASTROINTESTINAL TRACT OF THE WHITE RAT

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Recent investigations have indicated that cystine, long known as an amino acid containing sulfur present in proteins, and methionine, the second and more recently discovered sulfur-containing amino acid of the protein molecule, may function similarly in metabolism (1-3). While the physiological behavior of cystine has been the subject of frequent study, little is known concerning the metabolism of methionine. The oxidation of the sulfur of the methionine molecule has been studied in man (4), the dog (5), and the rabbit (6). The rate of absorption of cystine from the gastrointestinal tract of the white rat (7, 8) and from isolated intestinal loops of the dog (9) has been determined but no similar studies of the rate or extent of absorption of methionine have been reported. The present investigation is concerned with the absorption of *dl*-methionine from the gastrointestinal tract of the white rat as measured by the method used by Cori for the study of carbohydrate absorption. The glycogen content of the liver after the absorption of methionine has also been determined.

#### EXPERIMENTAL

In general, the experimental procedures used previously in this laboratory for the study of the absorption of the amino acids (10) have been followed. Male rats only have been used. In order to obtain results as nearly uniform as possible, rats were selected such that the weights after preliminary 24 hour fasting periods were approximately the same, 120 to 135 gm. The *dl*-methionine (synthetic) was fed as the sodium salt, since the solubility of the

methionine was so slight that solutions of the free acid of sufficiently high concentration for use in the absorption experiments could not be obtained. Moreover, in the previous studies of the absorption of cystine from the gastrointestinal canal of rats, with which we wished to compare the values obtained for methionine, cystine was administered as the sodium salt. The methods of preliminary treatment of the animal, administration of the amino acid, and of analysis of the gastrointestinal contents for amino acid nitrogen and of the liver for glycogen were the same as those previously reported (10). In all the experiments recorded, absorption of the amino acid was allowed to proceed over a period of 3 hours.

In the studies on cystine absorption (7, 8), it was not possible to determine the residual amino acid of the tract by the Van Slyke nitrous acid method, inasmuch as cystine reacts abnormally with nitrous acid. Since the  $\alpha$ -amino group of methionine has been observed to react quantitatively with nitrous acid in a manner similar to that of the other common amino acids, it was possible in our experiments to use the amino nitrogen in the gastrointestinal contents as a measure of the methionine remaining in the tract at the end of the absorption period.

It was necessary to establish normal values for the amino nitrogen contents of the gastrointestinal canal of the rat after short fasts. A series of twenty-one animals, treated in the same manner as were the experimental animals except that the control animals received 2 cc. of water instead of the solution of the amino acid, gave an average value of 7.9 mg. for the amino nitrogen of the tract, with individual determinations ranging from 5.0 mg. to 11.3 mg.<sup>1</sup> The average control figure has been used as a correction for all the experimental values presented in Table I.

The results of the absorption studies are presented in Table I, in which the rate of absorption of methionine is calculated per hour per 100 gm. of rat in terms of the amino acid, amino nitrogen, and milli-equivalents. In the first series (Series A), amounts of methionine corresponding to about 220 mg. per 100 gm. of body weight were fed. In order to determine whether the absolute amount of methionine present influenced the absorptive process, in a second series (Series B), the amount administered was in-

<sup>1</sup> The control values were very uniform, as only one value (11.3 mg.) was obtained greater than 9.4 mg. and only two less than 6.4 mg.

creased about 60 per cent. The results obtained were slightly lower than the average values of Series A, but we believe that they are within the limits of error of the experimental method.

Wilson (7), who used the Folin-Marenzi method for the determination of the residual cystine of the gastrointestinal contents,

TABLE I

*Absorption of dl-Methionine (Sodium Salt) from Gastrointestinal Tract of White Rat*

Series No	Rat No	Weight after fasting	Methionine		Rate of absorption per hr calculated in terms of		
			Fed	Recovered	Nitrogen	Amino acid	
		gm	mg per 100 gm rat	mg per 100 gm rat	mg per 100 gm	mg per 100 gm	m-eq per 100 gm
A	140	121	239	92	4.02	49	0 328
	141	121	239	80	5 03	53	0 359
	149	121	223	64	4 96	53	0 355
	151	126	220	46	5 44	58	0 389
	153	126	220	47	5 42	58	0 389
	152	127	218	62	4 89	52	0 349
	138	128	206	52	4 82	51	0 342
	139	131	201	33	5 28	56	0 376
	142	131	221	60	5 06	54	0 362
	148	133	205	70	4 20	44	0 295
	143	133	208	26	5 70	61	0 409
	150	134	203	44	4 99	53	0 355
Average					5 03	53	0 359
B	155	121	355	216	4 44	47	0 315
	154	122	352	200	4 72	51	0 342
	156	123	349	199	4 70	50	0 335
Average					4 62	49	0 331
" of Series A and B					4 95	52	0 353

obtained an absorption coefficient of 30.5 for cystine, a value comparable to that obtained by Sullivan and Hess (8) in a repetition of Wilson's experiments. However, when these latter investigators used other and, in their opinion, more specific methods for the determination of cystine, absorption coefficients of 51.5 (Okuda method) and 53.5 (Sullivan method) were obtained. These values



are similar to the absorption coefficients of methionine obtained in the present study. If these values are recalculated in terms of milli-equivalents of *cysteine* (i.e. 1 atom of sulfur, since methionine, with which we wish to make comparison contains only 1 atom of sulfur), the coefficients are 0.251 (Wilson), 0.425 (Sullivan and Hess by the Okuda method), and 0.441 (Sullivan and Hess by the Sullivan method). If the higher absorption coefficients are accepted as correct, the absorption coefficient of cystine (as milli-equivalents of cysteine) is slightly higher than that of methionine as observed by us (0.359). The absorption coefficient of methionine expressed in milli-equivalents is much less than that of glycine or alanine, slightly lower than that of glutamic acid, and slightly greater than that of leucine, if comparison is made with the values for the sodium salts of these amino acids previously obtained by one of us (10).

Analyses of the livers of the animals fed *DL*-methionine showed no evidence of the deposition of glycogen. The glycogen content of the livers of seven rats receiving methionine averaged 0.06 per cent with a maximal value of 0.22 per cent, figures of the same order of magnitude as the average glycogen content of the livers of fasting control rats in this series (0.05 per cent with a maximal value of 0.19 per cent).

The urine secreted during the 3 hour absorption periods was collected by placing the rats in small cages over funnels and was tested for the presence of compounds containing the —SS— linkage by the sodium cyanide-sodium nitroprusside reaction (11). In every case a positive test for the —SS— linkage was obtained. In this laboratory it has previously been observed (6) that the urines of rabbits to which methionine was administered reacted also in the cyanide-nitroprusside test to indicate the presence of a compound containing —SS— groups. Further evidence of the presence of compounds of this type in the urine of rabbits after administration of methionine was obtained. These findings and their probable significance in methionine metabolism will be discussed in another paper. In the experiments with rats, it was also observed that when the urines collected after methionine feeding were tested by the ammonium hydroxide-sodium nitroprusside test, a pink coloration developed which attained its maximum intensity in 7

to 8 minutes. We have never observed positive tests with either of these two color reactions in urines from normal rats.

The methionine used in these studies was the *dl* form. It would be desirable to compare with these results the results of a series in which the naturally occurring isomer, *l*-methionine, was fed. In former experiments with other amino acids in this laboratory (10, 12), we have not observed any significant differences in the absorption coefficients of the naturally occurring optically active and the synthetic racemic forms of the amino acids. It may also be pointed out that Jackson and Block (13) have reported that *d*-methionine, as well as the *l* form, was effective in the promotion of growth in rats fed a diet in which a low content of cystine was the chief limiting factor.

#### SUMMARY

1. The absorption coefficient of *dl*-methionine from the gastrointestinal tract of the young white rat was found to be 53 mg. per hour per 100 gm. of rat (0.359 milli-equivalent). This rate of absorption (expressed in milli-equivalents) was slightly lower than the rate previously observed for cystine (0.425 and 0.441 calculated as milli-equivalents of cystine) (8).

2. Under the experimental conditions, no deposition of glycogen in the liver could be observed after the absorption of *dl*-methionine over periods of 3 hours.

3. The urines of rats receiving *dl*-methionine orally contained a substance which gave a positive test for the —SS— linkage (cyanide-nitroprusside test).

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## PENTOSE METABOLISM

### III. THE RATE OF ABSORPTION OF *l*-RHAMNOSE AND THE FORMATION OF GLYCOGEN IN THE ORGANISM OF THE WHITE RAT AFTER ORAL ADMINISTRATION OF *l*-RHAMNOSE

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*l*-Rhamnose, the most common of the naturally occurring methyl pentoses, is found in many glycosides, the carbohydrate constituent of which is solely or chiefly rhamnose. A summary of the discovery, occurrence, and methods of preparation of rhamnose has been presented by Harding (1).

From the physiological point of view, the methyl pentoses are of considerable interest because of their possible relation to the hexoses. Oxidation of the methyl group to a primary alcohol would give rise to a hexose and if such a reaction occurred in the animal organism, the methyl pentoses might serve as sources of glucose and glycogen. *l*-Rhamnose, the naturally occurring optical isomer, by an oxidation of this kind, would be converted to *l*-mannose, the optical antipode of *d*-mannose, the natural form. No well controlled study of the absorption and glycogenic activity of rhamnose has been reported.

In view of these considerations, a study of the behavior of *l*-rhamnose after oral administration to the white rat has been undertaken. Miller and Lewis (2), in reporting a similar investigation with *d*-xylose, have summarized the literature connected with the conversion of the pentoses to glycogen. It should be pointed out that although the older evidence for glycogenesis from pentoses is not convincing, there is to be observed throughout the literature, a tendency to emphasize the superiority of rhamnose over the other common pentoses as a source of glycogen (3, 4). No

experiments, which are concerned with the glycogen deposition after rhamnose feeding, have been reported in which the method introduced by Cori (5) for the study of carbohydrate absorption and glycogenesis in fasting young white rats has been employed. The advantages of this method require no extended comment (2). A study of the influence of phlorhizin on the rate of absorption of rhamnose is also reported.

#### EXPERIMENTAL

The experimental methods of preliminary treatment of the animals, administration of the carbohydrate, slaughter of the animals, and analyses of the contents of the gastrointestinal tract for sugar and of the livers for glycogen were the same as previously described in the study of xylose (2). Male rats were used in all cases. Rats whose weights after fasting periods of 24 hours were from 100 to 125 gm. were preferred and were employed for most of the experiments.

Since rhamnose differs from both glucose and xylose in its reduction value in the Hagedorn-Jensen method of sugar determination, it was necessary to prepare a table of reduction values in terms of rhamnose. The reduction effected by known amounts of pure rhamnose was determined, the results were plotted, and from this curve, by interpolation, a table of reduction values of rhamnose for use with the Hagedorn-Jensen method was constructed. This table was then checked by a repetition of this procedure.

In one series of experiments, it was desired to determine glucose and rhamnose present together in pure solutions and in the protein-free filtrates obtained from the contents of the gastrointestinal tract. The reduction effected by the mixture was first determined by the Hagedorn-Jensen method. Fermentable sugar was then removed by the yeast treatment recommended by Somogyi (6) and the residual reduction was determined and calculated as rhamnose. The glucose was then calculated by difference, the unequal reduction values of glucose and rhamnose being taken into consideration in the calculation. This method applied to known mixtures of glucose and rhamnose gave satisfactory results.

Rhamnose in the urine was identified by the test with ammonium molybdate in the presence of sulfuric acid (7). In applying this test to rat urine, it was observed that undiluted urine (1 to 2

drops) gave a heavy greenish precipitate even in the absence of rhamnose. However, if the urine were diluted (1:5) and 1 to 2 drops of the diluted urine used for the test, the results were satisfactory. Tests carried out in this manner on normal urines, to which known carbohydrates were added, showed that of the carbohydrates studied (arabinose, xylose, rhamnose, fructose, glucose, and mannose), rhamnose alone gave the characteristic green color.

In the experiments with phlorhizin, the phlorhizin (Merck), recrystallized as suggested by Lusk (8), was injected subcutaneously (50 mg. in 0.5 cc. of olive oil) in the inguinal region for 4 successive days. During this period the animals received the usual stock diet of whole wheat bread, milk, and lettuce. After the period of 4 days of phlorhizin injection, food was withheld for 24 hours and the absorption of the carbohydrate and the glycogen formation were studied in the usual way.

Since the amount of carbohydrate absorbed from the gastrointestinal tract was calculated from the difference between the amount fed and that remaining in the tract, it was necessary to correct the latter value for the reducing power of the tract alone. Cori (5) in studies of carbohydrates which showed high coefficients of absorption considered that this correction was not significant. However, if the absorption coefficient is low, as in the present instance, a considerable error may be introduced if the correction for the normal reduction value of the tract is not made. One group of ten rats of an average weight of 121 gm. served as controls for the determination of the normal reduction values of the contents of the gastrointestinal tract after a 24 hour fast. Values of from 5.5 to 10.5 mg. with an average value of 7.2 mg. (calculated as rhamnose) were obtained. If the residual reductions were calculated as glucose, an average value of 5.6 mg. resulted. These corrections were applied in all the absorption studies.

In a series of check experiments, the accuracy of the experimental procedure was tested as follows. Rats were fed glucose in the usual manner, killed immediately without allowing any time interval for the absorption of the carbohydrate, and the contents of the tract were analyzed. In five experiments, in which glucose (585 to 590 mg.) was fed and the correction for the normal residual reduction was made, 98.4 per cent of the administered

TABLE I

*Absorption of l-Rhamnose after Oral Administration to Fasting White Rat and Effect of Injections of Phlorhizin Previous to Rhamnose Feeding upon Rate of Absorption*

Rat No	Weight after 24 hr fast	Absorption period	Rhamnose fed	Rhamnose absorbed		
				Total	Per 100 gm rat	Per 100 gm rat per hr.
	gm	hrs	mg	mg	mg	mg
49	100	1	268	42	42	42
50	101	1	268	41	40	40
41	104	1	283	44	43	43
38	107	1	550	52	49	49
48	111	1	269	41	37	37
40	113	1	550	62	55	55
43	118	1	283	52	44	44
44	159	1	239	31	19	19
Average	114			46	41	41
42	110	2	141	47	43	21
107	112	2	264	46	41	20
45	114	2	239	36	31	15
37	115	2	625	52	45	22
105	117	2	321	45	39	19
39	118	2	550	57	48	24
34	145	2	810	52	36	18
127	152	2	310	67	44	22
126	155	2	310	60	32	16
Average	126			51	40	20
128*	110	2	307	33	30	15
65	118	2	332	71	60	30
47	123	2	269	57	46	23
130	127	2	307	53	42	21
64	132	2	368	59	46	23
129	135	2	307	43	32	16
135	137	2	242	46	34	17
137	143	2	242	40	26	13
134	152	2	242	41	28	14
136	162	2	242	33	20	10
Average	134			48	36	18

\* These rats were given 1 cc. of the rhamnose solution at the beginning of the experiments and another cc. 1 hour later, and were killed 2 hours after the first dose.

TABLE I—*Concluded*

Rat No	Weight after 24 hr fast	Absorption period	Rhamnose fed	Rhamnose absorbed		
				Total	Per 100 gm rat	Per 100 gm rat per hr
	gm	hrs	mg	mg	mg	mg
46	108	3	239	45	41	14
36	113	3	810	51	46	15
32	119	3	281	31	26	8
33	121	3	281	53	44	14
27	144	3	570	47	32	10
66	144	3	338	54	38	12
67	144	3	338	40	28	9
29	155	3	775	55	35	11
Average	131			47	36	12
79†	110	2	313	27	24	12
58	119	2	368	23	20	10
77	123	2	313	24	20	10
69	127	2	330	20	16	8
85	130	2	443	38	29	14
61	142	2	330	28	20	10
59	171	2	337	15	9	5
Average	132			25	20	10

† This group received injections of phlorhizin daily for 4 days prior to the fasting period. Details are given in the text.

carbohydrate was recovered. In a further control of the experimental procedure, the absorption of glucose was studied. The absorption coefficients obtained, 239, 209, and 184 for periods of 1, 2, and 3 hours respectively, are comparable to the values of other workers (2, 5, 9-11).

The results of the absorption studies are presented in Table I. The absorption coefficient (mg. absorbed per 100 gm. of rat per hour) decreased as the period allowed for absorption was increased, *i.e.*, 41, 20, and 12 for absorption periods of 1, 2, and 3 hours respectively. These are lower coefficients than those of the common hexoses (5) and of xylose (2, 12). Cori (5) has reported a coefficient of 16 for arabinose over a 2 hour period, a figure comparable to the value obtained for rhamnose during a similar absorption period. Further study of the data of Table I shows that the



absolute amount of rhamnose absorbed per 100 gm. of rat was essentially of the same order of magnitude in all the experiments regardless of the duration of absorption. Thus the absolute values averaged 41, 40, and 36 mg. in 1, 2, and 3 hours respectively. Such results indicate that practically all of the rhamnose which disappeared from the tract must have been absorbed within the 1st hour and that little or no absorption occurred during the subsequent periods. In one series (Table I), the rhamnose was fed in divided doses, an hour apart, and the rats were killed after a total absorption period of 2 hours. Although the absorption per 100 gm. of body weight was slightly high in one experiment (Rat 65), in the other experiments, the values were within the range of the series of the other 2 hour absorption experiments.<sup>1</sup>

The fact that the absolute amount of rhamnose absorbed showed no increase over periods of absorption longer than 1 hour suggested that the presence of rhamnose might have resulted in some pathological changes in the intestinal mucosa which hindered further absorption. Magee and Reid (12) have shown that in cats anesthetized with amytal, arabinose and xylose in 0.75 M solution caused immediate and marked inhibition of all movements and shrinkage of the villi.<sup>2</sup> In order to secure evidence of any toxic action of rhamnose upon the intestinal membrane, rats were fed water, rhamnose, and glucose solutions respectively as in the absorption experiments. After 2 hours, the animals were killed and the upper part of the small intestine was examined microscopically. Dr. Carl V. Weller of the Department of Pathology, to whom we are indebted for the pathological examinations, reported that no morphological changes could be discovered to explain the failure of absorption of rhamnose.

Although no morphological changes were discovered by which the apparent lack of absorption could be explained, the possibility remained that rhamnose might have produced a functional change

<sup>1</sup> Most of the low values were obtained with rats of weights greater than 130 gm. (*e.g.*, Rats 134, 136, 137). We have observed repeatedly that the coefficients of absorption are more consistent if rats of 110 to 130 gm. of body weight are used in the experiments.

<sup>2</sup> The concentrations of the solutions of rhamnose used in our experiments ranged from approximately 0.7 M (Rats 44 and 45) to 2.4 M (Rats 34 and 36). In every instance except one, 2 cc. of the rhamnose solution were fed. Rat 42 received 1 cc. only.

in the intestinal membrane, which could not be demonstrated microscopically. In order to determine whether the absorptive power of the intestinal mucosa had been altered by the presence of rhamnose in the gut, a group of seven rats was fed a mixture of glucose and rhamnose and the rate of absorption of each sugar was determined after a 2 hour period (Table II). The determination of glucose in the presence of rhamnose has already been described.

TABLE II

*Rate of Absorption of Glucose and Rhamnose after Oral Administration of a Mixture of These Two Carbohydrates*

Rat No.	Weight after 24 hr. fast		Sugars fed	Sugars absorbed		
				Total	Per 100 gm. rat	Per 100 gm. rat per hr.
	gm.		mg.	mg.	mg.	mg.
86	107	Glucose	645	348	326	163
		Rhamnose	410	69	64	32
87	113	Glucose	587	521	461	230
		Rhamnose	350	53	47	23
91	105	Glucose	562	412	392	196
		Rhamnose	250	47	45	22
92	108	Glucose	562	458	424	212
		Rhamnose	250	47	43	21
104	108	Glucose	593	375	347	173
		Rhamnose	312	75	70	35
109	119	Glucose	670	443	373	186
		Rhamnose	262	65	55	27
110	116	Glucose	670	408	352	176
		Rhamnose	262	65	56	28
Average.	111	Glucose		423	382	191
		Rhamnose		60	54	27

In control experiments in which glucose was fed alone, absorption coefficients ranging from 161 to 269 with an average of 209 were obtained. When glucose was fed with rhamnose, the absorption coefficients of glucose ranged from 163 to 230 with an average of 191. This indicates that the ability of the intestinal membrane to absorb glucose was not significantly altered by the presence of rhamnose.<sup>3</sup> Cori (13), in experiments in which a mixture of equal

<sup>3</sup> Further evidence that the glucose was absorbed and metabolized normally when introduced into the gastrointestinal canal with rhamnose is

amounts of glucose and galactose was fed, observed that the rate of absorption of both these readily absorbed sugars was reduced to such an extent, that the total amount of sugar absorbed was not greater than if either sugar alone were presented for absorption. Since rhamnose is so poorly absorbed, its presence would not be likely to alter significantly the rate of absorption of the glucose. These experiments and the pathological examination of the intestine seem to indicate that rhamnose did not produce any toxic effect which changed the absorptive power of the intestinal mucosa of the rat.

McCance and Madders (14) made a comparative study of the rates of absorption of xylose, rhamnose, and arabinose in the white rat by the Cori method. They concluded that rhamnose was poorly absorbed. Inasmuch as male rats of 230 to 300 gm. were used and the weights of the individual animals used are not stated, it is impossible to make any more direct comparison of their results with our own. Four rats only were used in experiments of 3 hours duration.

It has been reported that under the influence of phlorhizin, the rate of absorption from the gut is altered. Wilson (11) in experiments in which the Cori procedure was used, showed that the rate of absorption of glucose in phlorhizinized rats was only 70 per cent of that of normal animals. It has been shown (12, 15) that glucose absorption in the white rat was increased by the presence of phosphates in the intestine, while the rate of absorption of the pentoses (xylose) was not altered. It was considered of interest to study the effect of phlorhizin on the absorption of rhamnose in order to determine whether the effect of phlorhizin on the absorption of a pentose varied from the effects of this glycoside on absorption of glucose. The results are presented in the last series of experiments of Table I. When rhamnose was fed to phlorhizinized rats, both the coefficient of absorption and the absolute amount of rhamnose absorbed were decreased to about 50 per cent

afforded by analyses of the glycogen content of the liver made on these same animals. The per cent of liver glycogen after the 2 hour absorption period ranged from 0.94 to 2.02 per cent with an average of 1.40 per cent. In control experiments in which glucose was administered alone, the average per cent of liver glycogen after a 2 hour absorption period was 1.43

of the normal value. The absorption of rhamnose and that of glucose are affected similarly by phlorhizin injections.

The glycogen content of the liver and in some animals that of the rest of the body were determined in the experiments detailed in Table I. In no case was the glycogen content found to be increased above the fasting level after the oral administration of rhamnose. This is presumably to be ascribed in part to the slow absorption and rapid excretion of the rhamnose after administration, but is undoubtedly related also to the difficulty in metabolizing the rhamnose. The urine of rats fed rhamnose reduced Benedict's alkaline copper solution and gave strongly positive tests for rhamnose even during the 1st hour of absorption. These observations are in harmony with those of former workers and indicate that absorbed rhamnose was rapidly excreted by the kidneys as a foreign substance.

A similar rapid excretion of pentose has been reported by Grafe (16) in experiments with dogs and men who observed that 40 to 50 per cent of the xylose fed was excreted by the kidneys. He obtained no evidence of glycogen formation after oral administration of xylose to dogs, a finding supported by recent studies of Magendantz (17) with dogs. Grafe, unable to show any difference in the utilization of xylose in normal and diabetic men, concluded that, "Daher bestehen für seine Verwendung beim Zuckerkranken als Zuckerersatz keine Bedenken."

It is of interest to compare the behavior of rhamnose with that of the other pentose, *d*-xylose, recently studied in this laboratory (2). The absorption of rhamnose in the initial period of absorption was greater than that of xylose. With one exception (Rat 44, Table I), the amount absorbed in 1 hour was greater than the highest value (34 mg.) obtained with xylose, while the average rhamnose absorption (41 mg.) during this period was definitely higher than the average obtained for xylose (29 mg.). The absorption of xylose, however, continued at a slightly greater rate during subsequent periods (2 and 3 hours) while the absorption of rhamnose occurred to a very limited extent, if at all, after the 1st hour. It is impossible for us to explain this difference in behavior in the light of our present information. We do not consider that it is to be ascribed to any toxic substance present in the rhamnose, used since absorption of glucose was shown to proceed normally in the

presence of the rhamnose. No evidence is afforded by these studies to indicate any superior utilization of rhamnose as compared with xylose (2).

#### SUMMARY

1. The absorption coefficients of *l*-rhamnose, the methyl pentose of most common occurrence in nature, from the gastrointestinal tract of the white rat, as determined by the method of Cori (5), decreased with the increased duration of the period of absorption; *i.e.*, absorption coefficients (mg. per 100 gm. of rat per hour) of 41, 20, and 12 for 1, 2, and 3 hour absorption periods respectively. The absolute amount of rhamnose absorbed per 100 gm. of rat was nearly constant in all the periods of absorption regardless of the duration of absorption, 41, 40, and 36 mg. respectively. This suggests that no significant absorption of rhamnose occurred after the 1st hour.

2. After oral administration of glucose in mixture with rhamnose, glucose was as well absorbed from the gastrointestinal canal as when glucose was fed alone. This is believed to indicate that the poor absorption of rhamnose is not to be ascribed to any toxic effects of the rhamnose fed.

3. In phlorhizinized rats, the rate of absorption of rhamnose was decreased to about 50 per cent of the normal value.

4. No evidence was obtained that under the experimental conditions, oral administration of rhamnose resulted in a deposition of glycogen in the liver or that rhamnose was superior to xylose as a precursor of glycogen.

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## THE OXIDATION OF THEELIN AND SOME THEELOL DERIVATIVES\*

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Continuing our studies on the chemistry of the estrus-producing hormones, we have endeavored to gain further insight into the constitution of theelin and theelol by oxidative degradation procedures, by means of which we hoped to obtain less complex molecular structures. We have obtained several compounds having 1 carbon atom less but when the degradation was carried farther than this the oxidation was rapid and extensive. In order to have some guide in our experiments and to minimize waste of material we first determined the "oxygen equivalents" of the compounds to be oxidized, using the method of Smith and Spoehr (1930). Oxygen equivalents were determined for theelol, theelol methyl ether, theelin, theelin methyl ether, and desoxotheelin in pyridine solution and in a few cases in aqueous alkaline solution. A few typical curves are shown in Fig. 1. These show that in general the extent of degradation of the molecule depends upon temperature as well as upon the strength of the potassium permanganate solution. Thus Curves I and II for theelol were obtained at the same temperature but the concentration of permanganate in the second case was considerably greater, resulting in a correspondingly greater oxygen consumption. For Curve III there was a slightly lower initial permanganate concentration than for Curve II but the temperature was 5° higher, and this likewise caused a greater oxygen uptake. Reference to the curves for theelin shows that the same holds true for that substance, as indeed it does for all

\* With the exception of the report on bioassay, all of the data contained in this paper were presented at the meeting of the American Society of Biological Chemists at Cincinnati, April 10-12, 1933.



compounds studied, with one exception. Only in the case of theelin methyl ether was a definite end-point apparent. Curves VII, VIII, and IX show that for this compound the values obtained for the oxygen equivalent do not vary with moderate changes in temperature and permanganate concentration. The

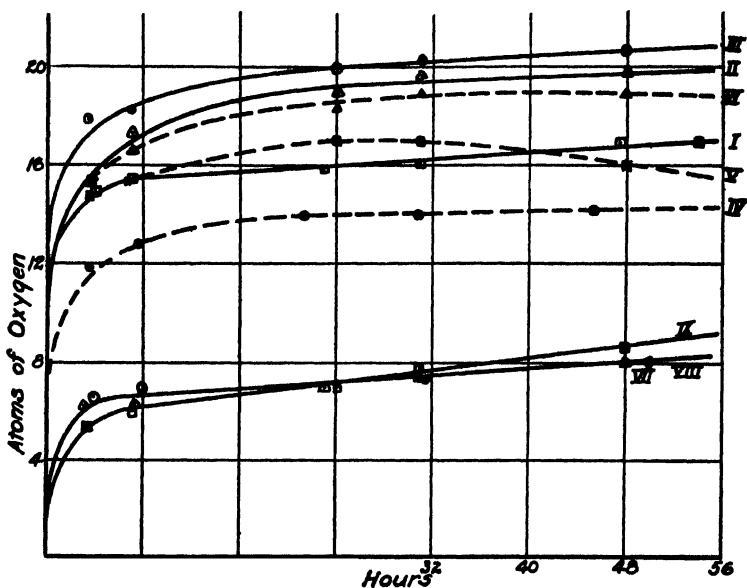


FIG. 1. The oxidation of theelinol,  $C_{18}H_{24}O_2$ , theelin,  $C_{18}H_{22}O_2$ , and theelin methyl ether,  $C_{18}H_{24}O_2$ . The theelin curves are shown in the broken line. Curve I, 16.7 mols of  $KMnO_4$  per mol of theelinol at  $30^\circ$ ; Curve II, 29.0 mols of  $KMnO_4$  per mol of theelinol at  $30^\circ$ ; Curve III, 27.0 mols of  $KMnO_4$  per mol of theelinol at  $35^\circ$ ; Curve IV, 15.9 mols of  $KMnO_4$  per mol of theelin at  $30^\circ$ ; Curve V, 15.7 mols of  $KMnO_4$  per mol of theelin at  $35^\circ$ ; Curve VI, 25.5 mols of  $KMnO_4$  per mol of theelin at  $35^\circ$ ; Curve VII, 20.0 mols of  $KMnO_4$  per mol of theelin methyl ether at  $30^\circ$ ; Curve VIII, 28.0 mols of  $KMnO_4$  per mol of theelin methyl ether at  $30^\circ$ ; Curve IX, 21.4 mols of  $KMnO_4$  per mol of theelin methyl ether at  $35^\circ$ .

low value for the oxygen equivalent shows that methylation of the phenolic hydroxyl stabilizes the aromatic nucleus against oxidation. The same was found true in the case of theelinol methyl ether. On the other hand, reduction of the carbonyl group of theelin to a methylene group did not stabilize the aliphatic ring

as the desoxotheelin was oxidized to approximately the same extent as theelin.

In order to detect any difference between the theelin obtained from human urine and from mare urine, we determined the oxygen equivalent of each under the same conditions but we could detect no difference, the results agreeing within the experimental error.

It has been shown (Marrian and Haslewood, 1932; MacCorquodale, Thayer, and Doisy, 1932-33) that fusion of theelol with caustic potash produces a phenolic dibasic acid,  $C_{18}H_{22}O_6$ , and that by treatment with methyl sulfate and alkali a monomethyl ether,  $C_{19}H_{24}O_6$ , of this acid is formed. We have subsequently found that the latter compound is also formed by the oxidation of theelol methyl ether with potassium permanganate in acetone solution. From the reaction mixture a second substance was also isolated having the formula,  $C_{18}H_{22}O_6$ . This substance proved to be the lactone of a monobasic hydroxy methoxy acid and appears to be formed by oxidation of the acid,  $C_{19}H_{24}O_6$ . We have also obtained two other substances by the oxidation of this methoxy dibasic acid. With aqueous potassium permanganate the oxidation is extensive but we succeeded in isolating a small amount of a methoxy dibasic acid having the composition,  $C_{19}H_{22}O_6$ . By chromic acid oxidation we have obtained a third compound which we found to be a methoxy lactone of the formula,  $C_{18}H_{18}O_6$ .

*Oxidation of Theelol Methyl Ether*—4.438 gm. of theelol methyl ether were dissolved in 800 cc. of dry acetone, and pulverized potassium permanganate was added to the solution in small portions as the oxidation proceeded. At first the reaction was slow but the speed of reaction increased after the oxidation was well started. When permanganate equivalent to 3 atoms of available oxygen had been consumed, the reaction slackened perceptibly and after 0.55 gm. additional had been added, the reaction stopped. After standing for 24 hours the supernatant liquid was colorless. It was separated by filtration and the residue washed with dry acetone. After distillation of the acetone from the water bath a small amount of a gummy residue remained which proved to be mainly impure theelol methyl ether.

The  $MnO_2$  residue was leached thoroughly with normal KOH solution and then with water. Acidification of these combined washings with dilute sulfuric acid precipitated a brown gummy

mass which gradually hardened on standing. It was washed thoroughly with water and dried. The weight was about 4 gm. It was dissolved in absolute alcohol and treated with an absolute alcoholic solution of potassium hydroxide. A light brown granular salt separated from the dark brown solution. It was filtered off and washed with absolute alcohol and then with dry ether. It was then dissolved in water and the solution acidified with hydrochloric acid, whereupon a brown gummy mass separated which gradually hardened on standing. This was washed thoroughly with water and was then dissolved in methyl alcohol and treated with norit. The filtrate, which still contained some color, was evaporated to dryness and the residue dissolved in hot glacial acetic acid. About 2 volumes of water were added to the hot solution, which on cooling deposited a brown crystalline solid. This was dissolved in methyl alcohol and decolorized with norit. Upon dilution with water and evaporation of some of the alcohol the solution deposited white crystals which when dried had a melting point of  $192^{\circ}$  (uncorrected). When mixed with a sample of the methyl ether of the acid obtained by potash fusion of theelol, the melting point was unchanged. The yield of product was 1.79 gm. The stability of this acid in the acetone-permanganate solution is presumably due to the insolubility of the potassium salt.

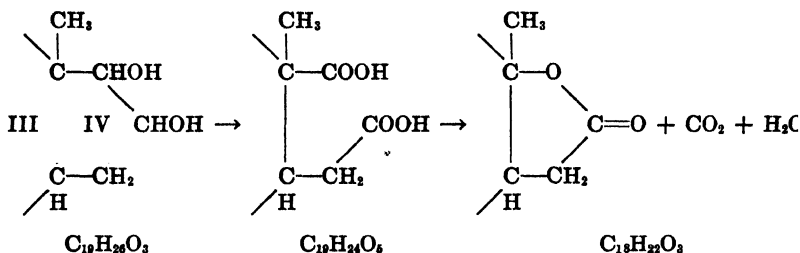
The alcoholic filtrate from the precipitated potassium salt was diluted with water and heated on the water bath to remove alcohol. Upon acidification with hydrochloric acid a brown gummy mass precipitated. This was washed, dried, and treated with methyl alcohol, whereupon most of the material dissolved, leaving a difficultly soluble crystalline mass. This material was washed with cold methyl alcohol and then dissolved in about 25 cc. of hot ethyl alcohol and decolorized with norit. The filtrate was concentrated to 4 cc. on the water bath, and on cooling deposited sheaves of long, well formed, needle-like plates. The yield was 175 mg. and the melting point  $182^{\circ}$  ( $178-179^{\circ}$ , uncorrected).

$C_{18}H_{22}O_8$ . Calculated. C 75.47, H 7.75,  $CH_3O$  10.81

Found. " 75.70, 75.84, H 7.60, 7.68,  $CH_3O$  10.76, 10.97

The substance was neutral to litmus and insoluble in cold 0.1 N alkali but slowly soluble on heating. As the neutralization equivalent could not be determined by direct titration, the material

was heated with an excess of standard alkali and the excess titrated with standard acid. The value found was 312 (theoretical 285). From alkaline solution the original compound was recovered by precipitation with acid. These properties and the analysis indicate that the substance is the  $\gamma$ -lactone of a monobasic acid. A crystallographic examination of the estrus-producing hormones by Bernal (1932) and studies of the surface films by Adam, Danielli, Haslewood, and Marrian (1932) have indicated that these compounds have a condensed ring structure, probably consisting of four rings, and the recent investigations of Marrian and Haslewood (1932), and Butenandt, Weidlich, and Thompson (1933) have shown that (IV), which in theelin bears the carbonyl group, and in theelol the aliphatic hydroxyl groups, is in all probability a 5-carbon ring. On this basis the formation of the lactone may possibly be represented as taking place in the following manner.



The location of the methyl group with respect to the hydroxyl groups seems fairly certain from the recent work of Butenandt, Weidlich, and Thompson (1933).

*Oxidation of the Acid,  $\text{CH}_3\text{OC}_{16}\text{H}_{19}(\text{CO}_2\text{H})_2$ , with Aqueous Permanganate*—572 mg. of the acid,  $\text{C}_{19}\text{H}_{24}\text{O}_6$  (melting point  $194^\circ$ , uncorrected), were dissolved with warming in a solution of 500 mg. of potassium carbonate in 50 cc. of water. At room temperature this solution was cautiously treated with a 0.1 N potassium permanganate solution, at first drop by drop with shaking, and then 10 cc. at a time, each time after the previous portion had been completely reduced. Toward the end the reaction slackened and the permanganate was added more rapidly. In all, 410 cc. were added. After standing at room temperature for 2 hours the solution contained only a trace of permanganate and this was reduced by warming on the water bath. The manganese dioxide was

removed by filtration and the filtrate concentrated on the water bath to a small volume and then acidified with dilute sulfuric acid. The brown resinous precipitate was dissolved in ethyl alcohol and treated with norit but it could not be induced to crystallize. It was then dissolved in absolute alcohol and treated with an absolute alcohol solution of potassium hydroxide. The brown potassium salt thus obtained was dissolved in water and the acid precipitated by means of dilute hydrochloric acid. Repeated crystallization of this from dilute alcohol finally yielded a small amount of white crystalline acid which melted at 234–235° (229–230°, uncorrected).

$C_{19}H_{22}O_6$ .	Calculated.	C 69.05, H 6.72, $CH_3O$ 9.4
	Found.	" 69.19, 68.60, H 6.6, 6.8, $CH_3O$ 9.5, 9.5

0.0101 gm. required 1.21 cc. of alkali (normality factor = 0.04985). The neutralization equivalent calculated for the dibasic acid,  $C_{19}H_{22}O_6$ , was 165; found, 167. The removal of 2 hydrogen atoms by oxidation with alkaline potassium permanganate was an unexpected result. It is not easy to detect a difference of 2 hydrogen atoms in these substances which are notoriously difficult to burn in the combustion apparatus and it may be that this acid is an isomer of the original methoxy dibasic acid. Because of the considerably higher melting point there can be no question that the product is different from the original acid. Neither acid gives a color with tetranitromethane. We regret that due to the very small amount of material obtained we are at present unable to state anything more definite concerning the nature of this substance.

*Oxidation of the Acid,  $CH_3OC_{16}H_{19}(CO_2H)_2$ , with Chromic Acid*—1.843 gm. of the acid,  $C_{19}H_{24}O_6$ , were dissolved in 50 cc. of glacial acetic acid and oxidized with a solution made by dissolving 5.445 gm. of potassium dichromate and 4.5 cc. of concentrated sulfuric acid in 50 per cent acetic acid and diluting to 50 cc. The oxidizing solution was at first added slowly at room temperature and after the reaction slackened it was added in 5 cc. portions and the mixture warmed on the water bath. The total volume of oxidizing solution used was 41 cc. The mixture was warmed on the water bath until the chromate was completely reduced and it was then diluted with about 4 volumes of water and set in the ice box to cool. The light brown granular precipitate which separated was

filtered off, dissolved in 75 cc. of 95 per cent alcohol, and treated with norit. The filtered solution was concentrated to 10 cc. and on cooling deposited fine white needles. The yield was 75 mg. and the melting point 243–244° (238°, uncorrected).

$C_{18}H_{18}O_8$ . Calculated. C 68.76, H 5.77,  $CH_3O$  9.87  
Found. " 68.94, 68.86, H 5.88, 5.99,  $CH_3O$  9.60, 9.90

The substance is neutral to litmus and insoluble in cold alkali but on prolonged heating it passes slowly into solution. It does not react with semicarbazide or with hydroxylamine. The neutralization equivalent was determined by heating the substance with an excess of standard alkali and titrating the excess base with acid. The value obtained, 175, indicates that the substance is the dilactone of a dihydroxy dibasic acid. The calculated value for the neutralization equivalent is 157.

*Fusion of Theelin with Caustic Alkali*—100 mg. of theelin from mare urine were fused with 5.0 gm. of potassium hydroxide and 0.75 cc. of water in a nickel crucible at 275° for 1½ hours. The brown fusion was dissolved in water and filtered, the filtrate saturated with carbon dioxide, and the white gelatinous precipitate separated by filtration. The filtrate was concentrated somewhat on the water bath and then acidified with concentrated hydrochloric acid. The white precipitate thus obtained was dissolved in 95 per cent alcohol, the solution decolorized with norit, and then concentrated on the water bath. On dilution with water and cooling, fine white crystals were obtained. Recrystallization of this product from dilute alcohol gave 20.5 mg. of fine white needles which melted sharply at 195° (193°, uncorrected).

$C_{17}H_{22}O_8$ . Calculated. C 74.40, H 8.09  
Found. " 74.49, 74.40, 74.24, H 8.51, 8.20, 8.10

The acid readily reddens litmus paper when in dilute alcoholic solution. Its phenolic character is indicated by the formation of an orange-red dye when its alkaline solution is treated with diazotized sulfanilic acid. Fusion of theelin from human urine produces the same acid. In their recent excellent publications, Butenandt, Weidlich, and Thompson (1933) have indicated that they have also carried out the alkali fusion of theelin and presumably they have likewise obtained this acid, although a description of their work has not yet appeared.

**Bioassays**—We are at present engaged in an extensive investigation of the physiological activity of the crystalline oxidation products mentioned above on immature and on ovariectomized rats and mice. The procedure for assay has been previously outlined by Kahnt and Doisy (1928). In addition to the subcutaneous we are also studying the enteral administration. Although our data are not complete, some highly interesting results lead us to make a preliminary report. We have found all of these substances to be considerably more active than theelin. However, in contrast to theelin a positive response is not obtained until the 4th or 5th day, and the vaginal smear remains positive during the 5th, 6th, and 7th days. Thus the potency of the acid,  $C_{18}H_{22}O_6$ , obtained by potash fusion of theelol was found to be greater than 6000 rat units per mg.—2 or 3 times that of theelin—the response being observed on the 5th day after administration and continuing through the 7th day. Both the methoxy dibasic acid,  $C_{18}H_{22}O_6$ , and the methoxy lactone,  $C_{18}H_{18}O_6$ , show an activity of about 20,000 rat units per mg. The phenolic acid,  $C_{17}H_{22}O_3$ , and the methoxy lactone,  $C_{18}H_{22}O_3$ , likewise show a greater activity than theelin.

In view of the fact that work on liquor folliculi (Thayer, Jordan, and Doisy, 1928) had shown that the activity was destroyed by peroxides present in the ether used for extraction, we were at first much surprised to discover the amazing potency of these oxidation products. It seems probable that the oxidation of theelin by the peroxides results in destruction of the aromatic nucleus, whereas the benzene ring is still present in these other oxidation products. One may conclude from this that the aromatic ring is essential for estrogenic activity.

#### SUMMARY

1. Quantitative determinations have been carried out on the permanganate oxidation of theelin, theelol, and some of the derivatives. In all cases except that of theelin methyl ether the extent of the reaction was found to vary with the temperature and with the concentration of the permanganate.

2. Methylation of the phenolic hydroxyl group in theelol and theelin stabilizes the aromatic nucleus against oxidation; reduction of theelin to the desoxo compound does not stabilize the ring which in theelin bears the carbonyl group.

3. No difference was found in the oxygen equivalents of theelin from human urine and theelin from mare urine.

4. Oxidation of theelol methyl ether with potassium permanganate in acetone solution produces a methoxy lactone of the formula,  $C_{18}H_{22}O_8$ , and an acid,  $C_{18}H_{24}O_8$ , identical with the methyl ether of the acid obtained by potash fusion of theelol.

5. By oxidation of the acid,  $C_{18}H_{24}O_8$ , with aqueous permanganate an acid was obtained with the probable formula,  $C_{18}H_{22}O_8$ .

6. By the oxidation of the acid,  $C_{18}H_{24}O_8$ , with chromic acid a methoxy dilactone of the formula,  $C_{18}H_{18}O_8$ , was obtained.

7. Fusion of theelin with potassium hydroxide produces a phenolic monobasic acid,  $C_{17}H_{22}O_3$ . Theelin from mare urine and that from human urine give the same substance.

8. These oxidation products have several times the estrogenic potency of theelin.

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## THE CHEMICAL COMPOSITION OF THE ACTIVE PRINCIPLE OF TUBERCULIN

### XVII. A COMPARISON OF THE NITROGEN PARTITION ANALYSES OF THE PROTEINS FROM DIFFERENT ACID-FAST BACILLI AND THE RELATIONSHIP TO BIOLOGICAL ACTIVITY\*

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At least four investigators, Tamura (1), Johnson and Brown (2), Campbell (3), and Johnson and Coghill (4), have studied the amino acid content of proteins isolated from the bodies of the tubercle bacillus. A high proportion of basic amino acids was found by all of them; otherwise there was nothing in the analyses to suggest an unusual type of protein. The Van Slyke method (5) had been used by all of these investigators except Tamura, who employed the method of Kossel and Kutscher (6). A comparative summary of their results is given by Wells and Long (7).

Similar analyses have not been reported upon protein isolated from the culture filtrate, tuberculin. In view of the extensive work that has been done upon tuberculin protein, such analyses should be made, since they might serve as a means for identifying that portion of the molecule most significant in the production of the skin reaction in tuberculous animals.

The following investigation was therefore undertaken, and comparisons made of analyses of the protein isolated by different methods and proteins isolated by one method from different strains of acid-fast bacilli. Only small quantities of a number of these fractions were available, and it was therefore not possible to use the macro-Van Slyke method, since at least 1 gm. of material is required for a single analysis. Because, as stated above, comparative rather than absolute values were desirable, the Thimann (8)

\* Aided by a grant from the National Tuberculosis Association.

method was chosen. In this procedure 0.1 gm. of protein, yielding 10 to 15 mg. of total nitrogen, suffices for a complete analysis, and less than half the time is required to make check analyses by this method than is necessary to complete a single Van Slyke analysis. Most of the results recorded in this paper, therefore, were obtained by means of this micromethod. A brief description of the method, as used, will be given. In most respects it is the method as outlined by Thimann.

The following directions serve for check analyses. Enough material to contain about 25 mg. of nitrogen is dropped into 30 cc. of boiling HCl (1 part of water and 1 part of concentrated HCl) and boiled for 24 hours under a reflux condenser. Any humin which separates out is centrifuged off, washed three times with 1 to 2 cc. of boiled distilled water (only boiled water is used throughout the analysis), and analyzed for nitrogen. The supernatant fluid and washings are made up to 50 cc., and 2 cc. samples of this solution are analyzed for nitrogen. From this result plus the nitrogen removed as humin the total nitrogen is calculated. All nitrogen determinations are made by the Parnas-Wagner modification of the micro-Kjeldahl method (9). The methyl red-methylene blue indicator of Tashiro (10) is used in all titrations.

From the remaining solution 20 cc. samples are used for check analyses for the nitrogen partition, as follows:

*Removal of Acid*—The acid is evaporated *in vacuo* at 50–60° in very small (30 cc.) Kjeldahl flasks fitted with a capillary reaching to the bottom of the tube and a small distillation bulb leading to a condenser and then to a receiving flask, to which in turn is connected the water pump. The small amount of air passing through the capillaries is first bubbled through  $\text{H}_2\text{SO}_4$  to remove ammonia. The liquid is brought as near to dryness as possible and then phenolphthalein is added.

*Amide Nitrogen*—2 cc. of 10 per cent NaOH are added, followed by 10 per cent  $\text{H}_2\text{SO}_4$  until the mixture is just acid. Then 2 per cent NaOH is added until the solution is just pink and 10 more drops are added to insure the pinkness throughout the determination.  $\text{NH}_3$ -free air is then bubbled through at 40–50° at a rate of about one bubble per second and at the same time as much vacuum is applied as is possible without endangering either the sample or the 25 cc. of 0.01 N  $\text{H}_2\text{SO}_4$  in the receiving flask. The

aeration is continued for 2 hours and the remaining acid titrated. Unless the results check within 1 to 2 per cent the procedure is repeated.

*Humin Nitrogen*—The solution is slightly acidified with  $\text{H}_2\text{SO}_4$  and all precipitates settling out during this determination are centrifuged off, washed, and analyzed for nitrogen. This value is added to the first humin fraction to obtain the total humin nitrogen.

*Basic Nitrogen*—The supernatant solution and washings are placed in a hard glass boiling tube ( $30 \times 210$  mm.) marked at 10 cc. and 0.3 cc. of concentrated  $\text{H}_2\text{SO}_4$  is added. The solution is evaporated to a small volume *in vacuo*, made up to 10 cc., and cooled in ice. 3 cc. of phosphotungstic acid solution (5 gm. of  $\text{H}_2\text{SO}_4$  and 20 gm. of phosphotungstic acid per 100 cc.) are added from a pipette, drop by drop, with shaking. After standing in ice overnight the precipitate is centrifuged off and well washed five times with a total of 10 cc. of phosphotungstic acid wash liquid (5 gm. of  $\text{H}_2\text{SO}_4$  to 2.5 gm. of phosphotungstic acid per 100 cc.), the suspension being centrifuged for 10 minutes each time, and then the liquid decanted. The washed precipitate from one of the two 20 cc. samples is then dissolved in 2 cc. of 10 per cent NaOH and washed quantitatively into a digestion flask and analyzed for total basic nitrogen.

*Non-Basic Nitrogen*—The supernatant fluid and washes from each phosphotungstic acid precipitate are combined and made up to 50 cc. and nitrogen is determined on 10 cc. aliquots.

*Arginine Nitrogen*—The phosphotungstic acid precipitate from the second 20 cc. sample is dissolved in 25 cc. of 40 per cent NaOH, and to it are added 25 cc. of water and 1 gm. of powdered zinc. The mixture is boiled for 6 hours under a reflux condenser at the top of which is a Folin urea absorption bulb containing 25 cc. of 0.01 N  $\text{H}_2\text{SO}_4$ . As recommended by Plimmer (11), the water is let out of the condenser and without disconnecting, the mixture is allowed to steam 15 to 20 minutes longer through the absorption bulbs, thus removing the last traces of released ammonia from the solution. The remaining acid is titrated; this gives the nitrogen representing half the amount of arginine present.

By cooling the boiling mixture, adding 27 cc. of concentrated  $\text{H}_2\text{SO}_4$  to it gradually and then a little  $\text{CuSO}_4$ , and digesting several hours after it has turned green in the usual Kjeldahl manner, the

total remaining nitrogen can be determined. This plus the nitrogen obtained from the arginine determination should give a figure for total basic nitrogen which should check the previous direct determination on the other sample.

In this study duplicate analyses checked within 5 per cent error. In most cases the differences were very much less.

Comparison of the analyses obtained by means of the macro-Van Slyke method and Thimann's micromethod as just described shows the same variations that were pointed out by the latter author; *viz.*, in the micromethod the amide nitrogen fraction is higher,

TABLE I  
*Comparison of Van Slyke and Thimann Micromethods*

Method used	Ghadin		TPA		TPU		TPA + polysaccharide
	Macro	Micro	Macro	Micro	Macro	Micro	Micro
Total N, per cent	17.2	17.2	16.2	16.2	3.8	3.8	3.5
Reducing substances (on hydrolysis), per cent	5.4	5.4	6.4	6.4	68.7	68.7	68.7
	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Total N							
Amide N.	23.8	27.0	9.4	12.3	23.3	31.3	16.5
Humin "	2.5	0.1	5.4	1.8	8.2	3.9	4.3
Total basic N	11.2	3.9	21.2	12.9	23.0	19.9	12.5
Arginine "	5.6		12.2	7.6	22.6		6.2
Non-basic "	57.6	69.8	63.2	75.8	40.2	47.4	70.4

TPA = tuberculin protein prepared by means of the ammonium sulfate method; TPU = tuberculin protein prepared by means of the ultrafiltration method.

the humin and basic fractions lower, and the non-basic higher (Table I).

The nitrogen recorded as amide nitrogen may not be all real amide nitrogen, but, as Vickery (12) has maintained, may arise partly from deamination of the amino acids. While the deviation, therefore, from absolute chemical values may be considerable when using such a micromethod, nevertheless, the results obtained under identical conditions, as was true in this investigation, have comparative value and should be considered only in such a manner.

Analyses were made for total sulfur by the perhydrol-nitric acid

method of Stockholm and Koch (13). While there is no justification for considering all of the sulfur as cystine sulfur, the sulfur found was calculated as cystine in order to determine the maximum possible amount of cystine nitrogen which could be present. The calculations for per cent of total nitrogen as cystine nitrogen led to figures as follows: 0.77 per cent for human TPA,<sup>1</sup> 1.52 per cent for timothy TPA, 1.14 per cent for avian TPA, and 0.93 per cent for bovine TPA. This is in strong contrast to 6.08 per cent for insulin as reported by Wintersteiner, du Vigneaud, and Jensen (15). The real amount of cystine nitrogen in these fractions was probably less than the figures given. In the macro-Van Slyke analysis of TPA (Table I) the cystine nitrogen, determined in the usual way, was 2.4 per cent of the total nitrogen. This amino acid is evidently not present in unusual quantity in tuberculin protein and bears no direct relationship to the biological potency of these products. It was, therefore, not determined directly in later analyses.

Gortner (16) and also Hart and Sure (17) showed that the presence of carbohydrate, and Hauge (18) that the presence of fat, caused considerable error in the Van Slyke analysis for the nitrogen partition of protein. Since polysaccharide has conspicuously accompanied the tuberculin protein in all isolations (19), a study of the effect of the presence of this polysaccharide upon the protein analysis was made, the data from which are given in Table I. In this case the pure polysaccharide (20) prepared by one of the authors from tuberculin, was added to the protein (TPA) in such an amount that the proportion of protein to polysaccharide was almost identical with that found in the fraction, TPU, which was the natural combination of the two components as it existed in the tuberculin before either one was separated from the other. The percentage of reducing substances was recorded in terms of

<sup>1</sup> Throughout this paper the abbreviations for tuberculin protein reactions follow the system described in detail in a former paper (14). They are as follows: TPA = tuberculin protein prepared by means of the ammonium sulfate method; TPT = tuberculin protein prepared by means of the trichloroacetic acid method; TPU = tuberculin protein prepared by means of the ultrafiltration method; OTT = fraction prepared from Koch's "old tuberculin" in the same way as TPT; SOTT = fraction prepared from an "old tuberculin" made on synthetic medium in the same way as TPT.

results obtained by means of the Hagedorn-Jensen method, following hydrolysis, as described in a recent paper (20).

The addition of the tuberculin polysaccharide to the pure protein did cause variations, as indicated above, especially an increased deamination, but it did not account for all of the differences

TABLE II  
*Nitrogen Partition Analyses of Various Tuberculin Protein Fractions*

	Human TPA	Human TPT			Bovine TPT	Avian TPT	Timothy TPT	OTT	SOTT	Fraction 86	Fraction H-1
		Lot 1	Lot 2	Lot 8							
Total N, per cent	16.2	14.6	14.7	14.5	15.9	15.8	16.4	14.0	13.4	15.4	13.2
Reducing sub- stances (on hy- drolysis), per cent	6.4	5.0	7.0	0.6	4.2	1.0	0.5	5.2	1.3	6.1	11.3
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Total N	12.3	14.0	10.4	11.4	12.5	14.3	8.9	6.8	12.0	9.1	11.9
Amide N	1.8	2.2	1.5	2.3	0.9	1.8	1.2	1.0	2.2	2.2	3.6
Humic "											
Total basic N	12.9	13.6	14.9	14.8	10.8	12.0	12.6	18.4	24.6	17.9	23.3
Arginine N	7.6	7.8	7.5	10.0	6.6	9.7	7.9	11.4	9.3	7.7	12.3
Non-basic N	75.8	71.4	74.3	74.3	73.5	70.7	77.3	70.5	64.2	73.1	60.0
	$\text{mg} \times 10^{-3}$	$\text{mg} \times 10^{-3}$	$\text{mg} \times 10^{-3}$	$\text{mg} \times 10^{-3}$				$\text{mg} \times 10^{-3}$	$\text{mg} \times 10^{-3}$	$\text{mg} \times 10^{-3}$	$\text{mg} \times 10^{-3}$
N in minimal potent skin dose	0.043	0.045	0.053	0.045				0.063	0.041	0.060	0.070

TPA = tuberculin protein prepared by means of the ammonium sulfate method; TPT = tuberculin protein prepared by means of the trichloroacetic acid method; OTT = fraction prepared from Koch's "old tuberculin" in the same way as TPT; SOTT = fraction prepared from an "old tuberculin" made on synthetic medium in the same way as TPT.

between the analyses of TPA and TPU. An explanation of this discrepancy must await future research.

The results listed under human TPT, Lots 1, 2, and 8 (Table II) show the range of variation in analyses that can be expected by this method between similar preparations made at different times by the trichloroacetic acid method. The error on check analyses of any one product, as stated before, was only 5

per cent, or less. Any value, therefore, within these ranges must be considered a normal value, unless some future work proves that these protein preparations themselves are mixtures. Only values considerably outside of these ranges can therefore be considered significant at present in the search for a potent portion of the active molecule. It is seen that there was a corresponding close agreement between these analyses and similar ones on the TPA fraction, a preparation differing from TPT in that it was isolated by means of ammonium sulfate instead of by trichloroacetic acid.

Table II furthermore shows that the proteins prepared in a similar way, *i.e.* by means of the trichloroacetic acid method (21), from the bovine, avian, and timothy bacillus culture filtrates, gave nitrogen partition analyses almost within the ranges found for the human tuberculin protein. There was, therefore, no significant chemical difference detected by this method between these proteins, made from different strains of acid-fast bacilli.

Two analyses were made of fractions isolated by the trichloroacetic acid method from "old tuberculin" that had been made exactly according to Koch (OTT) or by his method with the exception that Long's synthetic medium was used for growing the bacilli (SOTT). A significant variation from the normal ranges was found here in the basic nitrogen fraction. These figures in both cases were higher than the highest value found in the four analyses of human tuberculin protein TPT.

Similar high basic nitrogen values were found in the cases of Fractions 86 and H-1. The former was a coagulated product made by autoclaving TPA for 1 hour at 15 pounds pressure and again for 15 minutes. Fraction H-1 was a fraction which spontaneously sedimented out during ultrafiltration of 196 liters of tuberculin from human type tubercle bacillus Strain 119 on an 8 per cent guncotton-glacial acetic acid membrane (22). These high basic nitrogen values apparently are not related to the potency of the preparation, as can be seen when a comparison is made of the last items in each column (Table II).

Biological reactions are always much more uncertain, in spite of their frequent greater delicacy, than are chemical tests such as those recorded in this paper. It is believed, however, that the tests reported in Table II represent as accurate data as can be obtained by means of the biological skin test. The level of skin



sensitivity of the tuberculous guinea pigs was determined first, and then, after only those guinea pigs of equal sensitivity were selected, a comparison of the potency of the different products was made on the basis of mg. of nitrogen just necessary to cause a maximum skin reaction, with beginning necrosis, as was described in greater detail in a former paper (21). This was called the minimal potent skin dose. Considering the limits of error of such a test, the potencies of the different fractions did not vary significantly from one another, except in the cases of Fraction 86, which was very much less potent, Fraction H-1, which was somewhat less potent, and OTT, which was possibly only slightly less potent.

#### SUMMARY

Three different protein preparations (TPT) made from tuberculin produced by the human tubercle bacillus, and containing almost identical amounts of total nitrogen, varied within narrow limits in their content of various nitrogen fractions. No significant chemical differences were detected by this method between these proteins, made from the culture filtrates of human tubercle bacillus, and proteins similarly prepared from the avian and the bovine tubercle bacillus and the timothy-grass bacillus filtrates.

Analyses of two proteins made similarly from Koch's "old tuberculin" and of two other denatured tuberculin protein preparations, gave significantly high values for basic nitrogen, but since these fractions were less potent when used for skin testing tuberculous guinea pigs than the others here reported, tuberculin activity cannot be referred to this basic nitrogen fraction.

The micromethod for the determination of the Hausmann numbers of proteins as described by Thimann was used for comparing the nitrogen partition of the various tuberculin protein fractions. Compared with results obtained by the Van Slyke method, with the micromethod the amide and non-basic nitrogen fractions are high, and the basic nitrogen low.

The presence of much polysaccharide (isolated from tuberculin) causes an increase in the amide nitrogen figure, probably due to deamination of amino acids, but does not entirely account for the high amide and basic nitrogen results obtained in tuberculin fractions where large amounts of polysaccharide are found naturally associated with the protein.

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## THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

### XXXIV. ISOLATION OF A PIGMENT AND OF ANISIC ACID FROM THE ACETONE-SOLUBLE FAT OF THE HUMAN TUBERCLE BACILLUS\*

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#### INTRODUCTION

When the human type of tubercle bacillus, Strain H-37, is cultivated on the Long synthetic medium, the organism grows on the surface of the liquid in the form of a faintly cream-colored pellicle and when the washed cells are suspended in a mixture of alcohol and ether, the bacteria appear to be nearly white. The alcohol-ether extract assumes gradually a pale straw color but when the extract is concentrated the color becomes dark red. The acetone-soluble fat, prepared from the alcohol-ether extract, contains practically all of the coloring matter and it forms a dark reddish brown salve-like mass.

In a previous report on the chemical composition of the acetone-soluble fat a series of fatty acids was isolated and described (1). It was indicated in the same publication that the fat contained some pigment which appeared to act as an indicator and after the fat had been saponified some of the pigment was carried over into the liquid fatty acid fraction.

For the present investigation a larger quantity of the acetone-soluble fat was saponified and the pigment, as well as certain other constituents, was separated from the fatty acids, as will be de-

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† Holder of a National Tuberculosis Association Fellowship at Yale University, 1932-33.

scribed in the experimental part. We were primarily interested in the isolation of the pigment, but incidentally we obtained from the same fraction a small quantity of a colorless crystalline substance which was identified as anisic acid.

The purified pigment was obtained in the form of yellow prismatic crystals melting at 173–174°, and its composition corresponds to the formula,  $C_{11}H_8O_3$ . The substance is only slightly soluble in water but it is easily soluble in organic solvents, with the exception of petroleum ether, giving yellow-colored solutions. The pigment dissolves easily in dilute alkalis giving deep red-colored solutions; on acidification the color changes to yellow and, when the solutions are sufficiently concentrated, yellow needle-shaped crystals separate. When the acidified solutions are distilled with steam, the pigment volatilizes and a portion of it crystallizes in the condenser in the form of yellow needles. The red-colored salts are easily soluble not only in water but in alcohol and acetone and have not been obtained in definitely crystalline form. The free pigment crystallizes from ethereal solutions on the addition of petroleum ether in massive orange-yellow prisms and also from methyl alcohol on addition of water as light yellow prismatic crystals. The color of the product varies from bright yellow to orange-yellow, depending upon the size of the crystals.

The tubercle bacillus, Strain H-37, used in this work had been grown on the Long synthetic medium (2), in the Mulford Biological Laboratories, Sharp and Dohme, according to the standardized procedure that has been adopted for the cultivation of all acid-fast bacteria used in our investigations. It is certain, therefore, that all compounds contained in the bacillary extracts had been synthesized by the living cells from very simple substances of known constitution.

#### EXPERIMENTAL

The acetone-soluble fat had been prepared by the method described in an earlier publication (3), and it possessed the same properties, color, and perfume-like odor as previously noted (1). The fat, about 260 gm., was saponified by refluxing for 4 hours with an excess of alcoholic potassium hydroxide. The unsaponifiable matter was removed in the usual manner by extraction with ether, after which the fatty acids were isolated.

The pigment, together with anisic acid and certain other unidentified constituents, was separated from the crude fatty acids as described below. The fatty acids, which were of a very dark color, were dissolved in alcohol, neutralized with potassium hydroxide, diluted with water, and the solution was poured into an excess of aqueous lead acetate solution. The lead soaps were filtered off and washed with water. The filtrate and washings which had a fine deep red color were reserved for the isolation of the pigment. The lead soaps were suspended in ether and decomposed by shaking with very dilute cold nitric acid after which the ethereal solution was washed with water and concentrated to dryness. The fatty acids were again converted into lead soaps and the latter were filtered off and washed with water. The filtrate which was of a light reddish color was combined with the first red-colored aqueous filtrate.

The lead soaps were dried *in vacuo* and treated with ether. The insoluble portion was filtered off, washed with ether, and reserved for the isolation of the solid saturated fatty acids. The liquid fatty acids were isolated from the ether-soluble lead soaps and converted into methyl esters. An additional small quantity of pigment was obtained by washing the ethereal solution of the esters with dilute sodium carbonate solutions until the washings were colorless.

The esters of the liquid fatty acids were recovered from the ethereal solution and reserved for future investigations.

The red-colored alkaline extract mentioned above yielded on acidification and extraction with ether 0.9 gm. of a yellow semi-solid material from which the pigment was recovered by steam distillation as will be described later.

*Separation of Anisic Acid from Crude Pigment*—The filtrates and washings obtained from the lead soap precipitations were concentrated under reduced pressure to a convenient volume, after which most of the red color was removed by extraction with amyl alcohol. Since it was impossible to remove all of the red color with amyl alcohol, the solution was acidified with hydrochloric acid when the color changed from red to yellow and much lead chloride separated. After the lead chloride was removed, the yellow pigment was easily extracted with ether. The ethereal extract was washed free of hydrochloric acid with water, filtered,

and the ether was distilled off. The residue was a yellow oily substance which soon crystallized.

The red-colored pigment was removed from the amyl alcoholic solution by extraction with dilute potassium hydroxide. The alkaline solution on acidification and extraction with ether yielded a further quantity of yellow crystalline material. The two portions were combined and amounted to 1 gm. The substance was not homogeneous; both yellow and colorless crystals were present together with an oily product and the mixture possessed a strong phenolic odor.

The crude material was dissolved in alcohol and the yellow solution, which was acid in reaction, was neutralized with potassium hydroxide. The color changed to deep red. After the alcohol was removed in a vacuum desiccator, the residue was extracted with several portions of warm acetone, leaving a nearly colorless residue. The pigment was isolated from the acetone solution as will be described later.

The acetone-insoluble substance which weighed 0.3 gm. was dissolved in water and the solution was decolorized with norit. On acidification of the filtrate with hydrochloric acid, colorless crystals separated. After the solution had been cooled in ice water, the crystals were filtered off, washed with cold water, and dried *in vacuo*. The substance, which weighed 0.2 gm., was twice recrystallized from hot water. Very long, slender, colorless, prismatic needles were obtained which melted sharply at 184°, solidified at 168°, and remelted at 184°. There was no change in the melting point on recrystallizing the substance from ether or dilute alcohol.

*Titration*—0.1431 gm. of substance dissolved in 25 cc. of neutral alcohol with phenolphthalein as indicator required 9.30 cc. of 0.1 N NaOH.

$\text{CH}_3\text{OC}_6\text{H}_4\text{COOH}$ . Mol. wt. calculated, 152; found, 153

The properties, melting point, and molecular weight of the substance correspond to those of *p*-methoxybenzoic, or anisic acid. A specimen of anisic acid (Kahlbaum) was purified by crystallization from water and from ether. The crystal form was identical with that of our substance. The acid melted at 184°, and when mixed with our crystals there was no depression of the melting

point. The identification of our product as anisic acid was completed by the determination of methoxyl followed by the isolation of the demethylated acid which was found to be *p*-hydroxybenzoic acid.

*Methoxyl Determination (Zeisel)*—0.1000 gm. substance: 0.1580 gm. AgI  
 $\text{CH}_3\text{OC}_6\text{H}_4\text{COOH}$  (152).  $\text{CH}_3\text{O}$  calculated, 20.38; found, 20.88

*Isolation of p-Hydroxybenzoic Acid*—The reaction mixture from the methoxyl determination was evaporated to dryness under reduced pressure; water was added several times and the evaporations repeated in order to remove the hydriodic acid as completely as possible. The residue was dissolved in a little hot water, treated with norit, and the solution was filtered. Aggregates of compact needle-shaped crystals separated from the solution on cooling. The yield was 50 mg. After the substance had been recrystallized from water it melted at 214–215°, solidified at 175°, and remelted at 213–214°.

A specimen of *p*-hydroxybenzoic acid was prepared by demethylating anisic acid (Kahlbaum) with hydriodic acid, the reaction product being worked up exactly as described above. After two recrystallizations from water the substance melted at 214–215°. When mixed with our demethylated acid there was no depression of the melting point.

It is evident from the data presented that the colorless crystals obtained from the acetone-soluble fat were pure anisic acid and that pure *p*-hydroxybenzoic acid was formed on demethylation.

The anisic acid obtained represents only a minimum of the quantity originally present in the fat. We were mainly concerned with the isolation of the pigment from the saponification mixture and it is very probable that serious losses of anisic acid occurred during the preliminary operations.

*Isolation of Pigment*—As has been mentioned previously the separation of the anisic acid from the pigment was accomplished by extracting the mixed dried potassium salts with warm acetone. The red-colored potassium salt of the pigment was soluble in warm acetone. For the isolation of the pigment the acetone solution was evaporated to dryness. The residue was dissolved in water, acidified with sulfuric acid, and distilled with steam. The pigment volatilized slowly; some yellow needle-shaped crystals



collected in the condenser and the distillate was yellow in color. After most of the pigment had volatilized, the substance which had been isolated from the alkaline extract of the methyl esters of the liquid fatty acids was added to the flask and the distillation with steam was continued until the distillate was practically colorless.

The yellow crystalline product after it had been filtered off, washed with cold water, and dried *in vacuo* weighed 0.3 gm. The distillate, about 3.5 liters, was extracted with ether, and the ethereal solution, after being washed with water, was concentrated to dryness. A small amount of a yellow crystalline residue was obtained and united with the product mentioned above. The substance still contained various impurities and a small amount of an oily material adhered to the yellow crystals. A strong phenolic odor was noticed and on standing in a closed flask a small amount of colorless crystals sublimed in the upper portions of the flask. The admixed impurities were removed by treatment with petroleum ether in which the yellow crystals were insoluble. The crystals, after they had been filtered off, washed with petroleum ether, and dried, weighed 0.21 gm. The material soluble in petroleum ether has not been further investigated.

The crude crystals were dissolved in 15 cc. of ether and the solution was diluted with 3 volumes of petroleum ether. On standing and cooling compact orange-yellow prisms separated from the solution. The crystals were filtered off, washed with petroleum ether, and recrystallized in the same manner. The yield was about 0.17 gm. The substance melted at 173–174°, solidified at 164°, and remelted at 173–174°. Some of the substance was again recrystallized from a more dilute solution in ether by adding petroleum ether and cooling in a freezing mixture. Fine delicate prisms of a lighter yellow color were obtained but the melting point was the same as that recorded above.

It was noticed in every case that the heated portions of the melting point tubes turned red in color and some of the substance sublimed, forming beautiful yellow prisms in the cool upper part of the melting point tubes.

The substance was odorless and very soluble in the ordinary organic solvents with the exception of petroleum ether, giving yellow solutions from which no crystals separated on cooling. The alcoholic solution of the pigment when diluted with water

deposited some yellow needle-shaped crystals, but the best combination of solvents for recrystallization appeared to be a mixture of ether and petroleum ether. On moist litmus paper the substance showed a strong acid reaction. So far as we have been able to observe, all the salts of the substance are red in color and are easily soluble in alcohol and water. The addition of ferric chloride to an alcoholic solution of the pigment gave a red color. The ammonium salt could be precipitated as a red amorphous powder by saturating an ethereal solution of the pigment with dry ammonia gas and this compound partly crystallized from a concentrated solution in alcohol.

In chloroform solution the substance showed no optical rotation.

*Microanalysis*<sup>1</sup>—3 509 mg. substance: 1.31 mg. H<sub>2</sub>O and 9.03 mg. CO<sub>2</sub>

C<sub>11</sub>H<sub>8</sub>O<sub>3</sub> (188). Calculated. C 70 21, H 4 25

Found. " 70 19, " 4 18

*Molecular Weight*—0 212 mg., 0 197 mg. substance; 1 376 mg., 2 934 mg. camphor; 30 5°, 13° depression of the melting point

C<sub>11</sub>H<sub>8</sub>O<sub>3</sub>. Mol. wt. calculated, 188; found, 202, 206

The values found for the molecular weight by the Rast method were too high but an accurate value by this method could hardly be expected owing to the volatility of the substance.

The constitution of this pigment will be reported in a subsequent paper.

#### SUMMARY

The pigment which is present in the acetone-soluble fat of the human tubercle bacillus, Strain H-37, has been isolated. It was obtained in the form of yellow prismatic crystals which melted at 173–174°. The composition corresponds to the formula, C<sub>11</sub>H<sub>8</sub>O<sub>3</sub>.

The pigment forms salts with various bases. The salts are all red in color and are easily soluble in water, alcohol, or acetone.

A colorless crystalline substance associated with the pigment fraction has been identified as *p*-methoxybenzoic acid.

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<sup>1</sup> We are indebted to Professor H. T. Clarke of Columbia University for the microanalytical determinations.



## THE RELATIONSHIP BETWEEN GASTRIC SECRETION AND THE ALKALINE TIDE IN URINE

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The theory that the alkaline tide in the urine is caused by the formation of hydrochloric acid in the stomach is one which has been advocated by many physiologists, but which has not been universally accepted. Since the literature dealing with the subject has been discussed by Munford and Hubbard (1926), Rannenberg (1926), and Davies (1929), it will not be reviewed further at this time. The authors believe that the question can best be studied by direct comparison of results upon normal subjects with those obtained under similar conditions upon patients with achlorhydria. A number of investigations of this type have been reported. While in most instances quite distinct differences have been noted between results on the two types of cases, such differences have not been invariably found (Watson, 1925; Hubbard and Case, 1930). It seems worth while, therefore, to report the rather extensive series of experiments described below.

This series is based upon the study of 273 sanitarium patients. None of them, as far as was known, was suffering from acute illness, apart from conditions closely associated with gastric secretion, and very few of them showed any signs whatever of cardiovascular-renal disease. The cases were selected and grouped for study on the basis of gastric analyses carried out by the fractional technique of Lyon, Barth, and Ellison (1921). These tests showed that in 125 hydrochloric acid was present in the gastric juice in approximately normal amounts, and that in 86 no acid was found. There were also two groups which were hard to classify in a satisfactory manner. The stomachs of twenty-three

patients apparently secreted less than the normal amount of acid, for either hydrochloric acid was present in less than half of the specimens obtained during the 105 minutes of the test or else the concentration of acid in all specimens was low, for titration to the end-point with Toepfer's reagent gave values less than 10 cc. of 0.1 N acid per 100 cc. of gastric juice in each specimen. The thirty-nine cases in the fourth group have also been classified as hypochlorhydria, although in some respects a diagnosis of achlorhydria might seem appropriate, as no specimen from them gave a positive reaction when tested with Toepfer's reagent. However, the total acidity determined by titration to the phenolphthalein end-point increased after the gastric test meal and reached values greater than 20 cc. of 1.0 N acid per 100 cc. of gastric juice in at least one specimen. Such increases probably do not take place unless some hydrochloric acid is secreted by the stomach. Since most of the errors in gastric analysis lead to results which are too low, patients upon whom a gastric analysis was carried out more than once have been classified according to the highest values obtained. Since histamine was not used in these studies, it is quite probable that some cases have been incorrectly called achlorhydric.

The reaction of the morning urine of these patients was studied on a different day from that on which the gastric analysis was carried out. The technique approximated physiological conditions closely, and was so simple that deviations from the regimen were infrequent. Each test began at 7 o'clock in the morning when the patient emptied his bladder. This specimen was discarded and hourly specimens collected thereafter until 1 o'clock. A meal consisting of a glass of milk, a glass of water, two slices of toast with butter, and an egg was fed between 8 and 9 o'clock. No other food was eaten during the test and water was withheld as far as was practicable. The reaction of each specimen was determined to the nearest 0.1 pH by a colorimetric method soon after it was voided. Since it was not practicable to take the precautions recommended by Marshall (1922), the loss of carbon dioxide undoubtedly caused some irregularity in the results.

The averages of all the results obtained are given in Chart I. These averages are based upon the number of persons studied and not upon the number of tests run, for when more than one test

was carried out upon a single patient, the results of all of them were averaged and the averages used in computing the values given. The chart shows clearly that there were marked differences between the results upon the two large groups of cases, and that those obtained upon the two small groups considered by us to be made up of patients with hypochlorhydria were essentially intermediate between the others. All the average pH values of

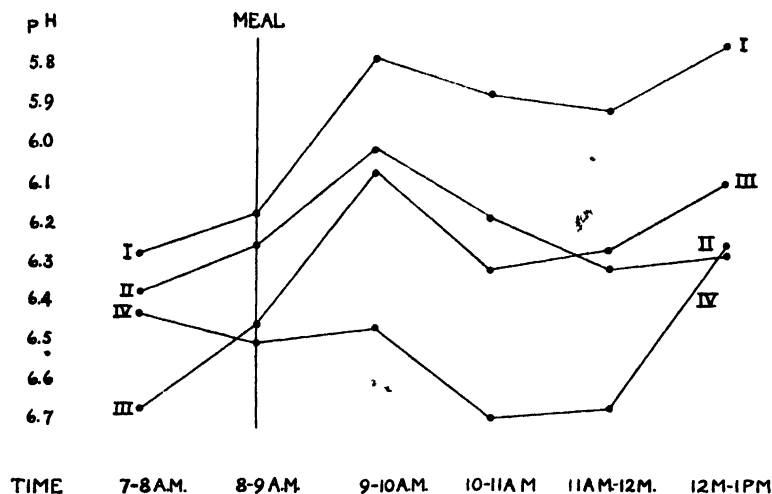


CHART I. Curve I is based upon the average values for 86 subjects in which hydrochloric acid was absent from the gastric juice; Curve II is based upon the average values for thirty-nine subjects with traces of acid probably present in the gastric juice; Curve III is based upon the average values for twenty-three subjects with small amounts of acid in the gastric juice; Curve IV is based upon the average values for 125 subjects with normal amounts of acid in the gastric juice. The details of the method of classifying results are given in the text.

the other specimens collected from patients with achlorhydria were more acid than was the first one, while a majority of those from subjects with normal gastric juice was more alkaline than was the one obtained an hour after waking. The urine of the patients with normal gastric findings showed a well marked alkaline tide after the test breakfast which was absent in the control series of patients with achlorhydria. This evidence supports the

thesis that secretion of acid by the stomach causes the alkaline tide in the urine.

These average figures also show quite clearly the effect of the morning respiratory tide which has been previously discussed (Leathes, 1920; Hubbard, 1929), for the patients with achlorhydria showed an increase in the urinary acidity as the relative alkalinity found immediately after awakening (Hubbard, 1930) passed off. Chart I suggests that there was possibly overcompensation for this early period of alkalinity, for the urine of the achlorhydric subjects showed a fairly well marked acid peak during the 3rd hour of the test. It is quite possible, however, that this peak has no physiological significance, for since histamine was not used in differentiating the cases of achlorhydria, some patients may have been incorrectly included in this group, and the results upon them have so influenced the average figures as to cause the slight increase in alkalinity shown during the 4th and 5th hours. The authors believe that increases in acidity similar to those just discussed were not shown by the patients with normal stomach contents because they were obscured by the effect of gastric secretion induced by the meal.

#### SUMMARY

Comparison of results upon two extensive series of patients, one with normal gastric contents and one with achlorhydria, shows that the secretion of hydrochloric acid by the stomach is the main cause of the alkaline tide after the morning meal. There are a number of conditions which may affect either the reaction of the urine or the secretion of hydrochloric acid by the stomach, and the effect of such factors probably explains the lack of a relationship between gastric acidity and urinary reaction which has been reported from time to time. There is a fairly regular increase in the acidity of the urine of patients with achlorhydria which probably represents, in part at least, recovery from an early morning period of alkalinity due to respiratory causes.

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Similarly, physiological phenomena such as *Absorption*, *Assimilation*, *Digestion*, *Equilibrium*, *Excretion*, *Fermentation*, *Metabolism*, *Respiration*, etc., are not indexed as such, but only under the substance in question.

When a definite constituent is given in connection with *Biological fluids*, *Biological material*, *Blood*, *Blood cell*, *Blood plasma*, *Blood serum*, *Diet*, *Milk*, *Tissues*, *Urine*, the constituent only is indexed; i.e., *Cholesterol in blood*, etc., is indexed under *Cholesterol*, not under *Blood*, etc. However, *Blood sugar* is indexed as such.

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